نموذج رقم (1)

نموذج التفويض

الجامعة الأردنية

نموذج التفويض

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نموذج رقم (١٦)
اقرار والتزام بالمعايير الأخلاقية والأمانة العلمية وقوانين الجامعة الأردنية وأنظمتها وتعليماتها لطلبة الدكتوراة

أنا الطالب: جميل فياض البزور الرقم الجامعي: (٩٠٥٠٠٧٣)

تخصص: العلوم الحياتية الكليـــــة: العلوم

عنوان الاطروحة: تقييم الفاعلية المضادة للروماتيزم لنبات الميرمية (العائلة الشفوية)، بإجراء دراسات على المستوى الإنزيمي من الخلوي في الأجسام الحية.

اعلن بأنني قد التزمت بقوانين الجامعة الأردنية وأنظمتها وتعليماتها وقراراتها السارية المفعول المتعلقة باعداد اطروحات الدكتوراه عندما قمت شخصيا" باعداد اطروحتي وذلك بما ينسجم مع الأمانة العلمية وكافة المعايير الأخلاقية المتعارف عليها في كتابة الأطروحات العلمية. كما أنني أعلن بأن اطروحتي هذه غير منقولة أو مستلة من أطاريح أو كتب أو أبحاث أو أي منشورات علمية تم نشرها أو تخزينها في أي وسيلة اعلامية، وتأسيسا" على ما تقدم فانني أتحمل المسؤولية بأنواعها كافة فيما لو تبين غير ذلك بما فيه حق مجلس العمداء في الجامعة الأردنية بالغاء قرار منحي الدرجة العلمية التي حصلت عليها وسحب شهادة التخرج مني بعد صدورها دون أن يكون لي أي حق في التظلم أو الاعتراض أو الطعن بأي صورة كانت في القرار الصادر عن مجلس العمداء بهذا الصدد.

توقيع الطالب: جميل فياض البزور التاريخ: ٢٠ / ٧ / ٢٠١١

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ASSESSMENT OF THE ANTIRHEUMATIC ACTIVITY OF *SALVIA FRUTICOSA* MILL. (SYN.: *SALVIA TRILOBA* L. FIL.) (LAMIACEAE); BY PERFORMING ENZYMATIC, CELLULAR AND *IN VIVO* STUDIES.

By
Jameel Fayyad Al-Bzour

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Dr. Sawsan A. Oran, Prof.

Co-Supervisor
Dr. Yasser K. Bustanji

Co-Supervisor
Dr. Mohammad K. Mohammad

This Dissertation was Submitted in Partial Fulfillment of the Requirements for the Doctor of Philosophy Degree in Immunology

تعتمد كلية الدراسات العليا
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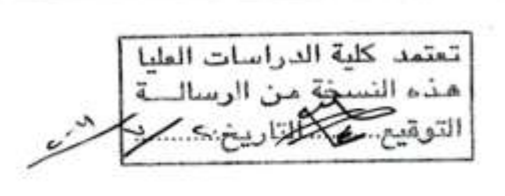
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II

COMMITTEE DECISION

This Thesis/Dissertation (Assessment of the Antirheumatic Activity of *Salvia fruticosa* Mill. (syn.: *Salvia triloba* L. fil.) (Lamiaceae); by Performing Enzymatic, Cellular and *in vivo* Studies) was Successfully Defended and Approved on 29 June 2011

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Dedication

With Love, Respect, Appreciation and Faithfulness, I dedicate this Thesis to:

My Dear Mother and Father

I could not thank them better than saying:

"My Lord! Bestow on them Your Mercy as they did bring me up when I was small." (Holly Qur'an- Surah Al-Isra', verse, 24)

Special dedication is also to my wife Eman and all my kids:

Hamza, Shaker, Omar, Tala, and Mohammad

To my brothers and sisters, especially my sister Asma'a

And to all of my supervisors, teachers, friends and faculties

For giving me their support and the power to proceed

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List of Contents

All Rights Reserved - Library of University of Jordan - Center of Thesis Deposit

All Rights Reserved - Library of University of Jordan - Center of Thesis Deposit

All Rights Reserved - Library of University of Jordan - Center of Thesis Deposit

All Rights Reserved - Library of University of Jordan - Center of Thesis Deposit

LIST OF TABLES

All Rights Reserved - Library of University of Jordan - Center of Thesis Deposit

LIST OF FIGURES

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LIST OF ABBREVIATIONS

AA	**Adjuvant Arthritis**
AAMQ	**Alternatively Activated Macrophages**
ABTS	**2,2'- Azino- Bis (3-ethaylbenzothiazoline-6- Sulfonic acid)**
ACPA	**Anti Citrullinated peptides Antibodies**
ACR	**American College of rheumatology**
AIA	**Adjuvant Induced Arthritis**
BSCF-2	**B Cell Stimulatory Factor-2**
BSA	**Bovine Serum Albumin**
CAM	**Complementary Alternative Medicine**
CD	**Cluster of Differentiation**
CIA	**Collagen- Induced Arthritis**
CFA	**Complete Freund's Adjuvant**
DAMPs	**Dammage- Associated Molecular Pattern molecules**
DAMARDs	**Disease- Modifying Anti Rheumatic Drugs**
DMSO	**Dimethyl Sulfoxide $(CH_3)_2SO$**
EDTA	**Ethylenediaminetetra acetic Acid**
FDA	**Food and Drug Administration**
FLS	**Fibroblast- Like Synovial cells**
gm	**Gram**
GM- CSF	**Grannlocyte/ Macrophage Stimulating Factor**
GPCRs	**G- Protein Coupled Receptors**
HLA	**Human Leukocyte Antigen**
HSF	**Hepatocyte Stimulating Factor**
ICE	**Interleukin- 1- Converting Enzyme**

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IFN- β_2	**Interferon- β_2**
IκB	**Inhibitor of $_{K}$B**
IKK	**I κB Kinase**
IL	**Interleukin**
IL-6 Rα	**Interleukin- 6 Receptor α**
INF-γ	**Interfron- γ**
JAKs	**Janus Kinase**
Kg	**Kilogram**
LPS	**Lipopolysaccharide**
MCP-1	**Monocyte Chemoattractant Protein-1**
M-CSF	**Macrophage Colony Stimulating Factor**
Mg	**Milligram**
MGI- 2 A	**Macrophage Granulocyte Inducer type2**
MHC	**Major Histocompatibility Complex**
MIP- 1α	**Macrophage Inflammatory Protein- 1**
MLS	**Macrophages – Like Synoviocytes**
MMPs	**Matrix Metalloproteinases**
mTOR	**Mammalian Target of Rapamycin**
MTT	**3-(4,5- dimethyl- 2- thiazolyl) 2,5- diphenyl- 2H- tetrazolium**
Mg	**Microgram**
NFκB	**Nuclear Factor Kappa B**
NLRs	**Nucleotide binding and oligomerization domain (nod)- like receptors**
NSAIDs	**Nonsteroidal Anti-Inflammatory Drugs**
OPG	**Osteoprotegerin**

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PAMPs	**Pathogen- Associated Molecular Pattern molecules**
pg	**Picogram**
PH	**Pleckstrin- Homolgy**
PI3Ks	**Phosphoinositide 3- Kinases**
PIP_2	**Phosphatidylinositol (4,5)- biphosphate**
PIP_3	**Phosphatidylinositol(3,4,5)-triphosphate**
RA	**Rheumatoid Arthritis**
RANKL	**Receptor Activator of NF-κB Ligand**
RF	**Rheumatoid Factor**
rpm	**Round per Minute**
RTK	**Receptor Tyrosine Kinases**
SLE	**Systematic Lupus Erythematosus**
STAT	**Signal Tranducer and Activator of Transcription**
TCA	**Trichloro acetic acid**
TCR	**T cell Receptor**
TCM	**Traditional Chinese Medicine**
TNF-α	**Tumor necrosis Factor- α**
Treg	**T- regulatory lymphocytes**
TGF-β	**Transforming growth factor β**
Th	**T helper cells**
The EMBO Journal	**The European Molecular Biology Organization Journal**
TLRs	**Toll-Like Receptors**
WHO	**World Health Organization**

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Abstract

Inflammatory arthritis, in particular rheumatoid arthritis (RA), is a chronic inflammatory disease which is an important health problem for millions of people worldwide. It has been the focus of intense investigation, but its etiology and pathogenesis remains controversial.
The current pharmacological treatment continues to be inadequate in preventing the progression of this disease to the stage of irreversible joint erosion and deformity.
Therefore, development of new anti-inflammatory and anti-rheumatic drugs continues to be essential. There is growing interest in the pharmacological potential of natural products. *Salvia* are being used extensively in folk medicine in Jordan and many other Mediterranean countries and this study aimed to investigate the ability of *S. fruticosa* to inhibit pro-inflammatory cytokines production, inhibit Phosphoinositide 3- Kinase (PI3K) enzyme activity and finally prevent the disease itself.
We first examined the inhibitory effects of Greek or Mediterranean sage *S. fruticosa* methanolic crude extract on TNF-α, IL-1β and IL-6 production in different murine cell types as *in vitro* cellular models and in LPS-challenged Balb/c mice as an *in vivo* animal model.
The results obtained showed a potent inhibitory effect of *S. fruticosa* on the production level of pro-inflammatory cytokines in both cellular and animal models. Also, PI3K enzyme activity was significantly suppressed by *S. fruticosa* intervention.
Finally, adjuvant-induced arthritis in rats was used as a recognized model represents a systemic inflammatory disease with bone and cartilage changes similar to those observed in humans RA. Rats treated with *S. fruticosa* showed no clinical manifestations, no significant increase in paw volume and no significant radiological

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observations related to development of arthritis. Moreover, there was no significant difference between treated group and normal control group. Whereas, the arthritic group showed all clinical manifestations, increased paw volume and typical radiological findings of subchondral sclerosis, decreased joint space and soft tissue swelling which are the hallmarks of RA, in addition to behavioral changes related to the painful nature of the disease.

It seems likely that *S. fruticosa* may act at various therapeutic targets in RA. Thus, and since that *S. fruticosa* has long been described traditionally, for treatment of many disease conditions. It can be concluded that *S. fruticosa* may offer a promising anti-inflammatory and anti-rheumatic agent for drug discovery researches. More experimental and clinical trials are also needed to validate the usefulness of its use either alone or in combination with existing therapy.

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1. Introduction

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder that most commonly affect the small joints of hands, feet and cervical spine. Larger joints like shoulder and knee can also be involved (Majithia and Geraci, 2007).

The synovium is relatively acellular structure in normal conditions, while there is an increased cellular infiltration, synovial hyperplasia, pannus formation and erosion of cartilage and bone in the distal joints in RA. Rheumatoid arthritis is estimated to occur in 0.5-1% of the general adult population worldwide (Firestein, 2003). The prevalence of RA among the first-degree relatives of patients with RA ranges from 2-12% (Dieude, 2009), and is higher in female patients over 40 years old (Deng and Lenardo, 2006).

Usually, the clinical symptoms of RA are swollen, red and pain joints with radiological evidence of joint deformation and bone destruction. Also, rheumatoid arthritis patients can experience severe loss of joint function in late stages without treatment. Although the etiology of RA remains uncertain, but there are hallmarks of autoimmunity (Deng and Lenardo, 2006).

Multiple mechanisms are proposed to be involved in synovial inflammation and an overlap between different pathways can occur. Synovial inflammation may be initiated by activation of innate immunity as well as many factors that may lead to T cell activation and B cell maturation. A combination of various events such as genetic background, innate and adaptive immune responses might cooperate in the progression of the disease process. However, in RA, the synovial lining is greatly thickened as the result of proliferation of synovial fibroblasts, infiltration of T and B

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lymphocytes, plasma cells and monocytes/macrophages (Firestein and Zvaifler, 2002).

1.2. Pro-inflammatory Cytokines and Nuclear Factor kappa (NF-κB) in RA

Many cytokines and chemokines, such as interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), IL-6, granulocyte/macrophage colony stimulating factor (GM-CSF) and macrophage inflammatory protein 1α (MIP-1α) are produced in high levels by activated macrophages in RA synovial tissue. The cytokine networking evokes the development of chronic inflammation and motivates bone destruction by the recruitment of osteoclast precursors and their differentiation into mature functional osteoclasts (Ma and Pope, 2005).

The genes encoding TNF-α and many of other inflammatory mediators are now known to be under the control of NF-κB transcription factors. The presence of activated NF-κB transcription factors has been demonstrated in cultured synovial fibroblasts, human arthritic joints and the joints of animals with experimentally induced RA (Simmonds and Foxwell, 2008).

1.3. Phosphoinositide-3-kinase (PI3K)

Intracellular signaling pathways have been engaged in the various stages of the inflammation and joint destruction process that characterizes RA. Due to its widespread activation in inflammation and cancer, a growing realization of the therapeutic potential of inhibitors of the PI3K pathway has encouraged great interest

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in compounds with suitable pharmacological profile. These are primarily directed toward PI3K itself (Crabbe *et al*., 2007).

Phosphoinostide-3-kinases are activated by receptors for antigens, cytokines, costimulatory molecules, immunoglobulins and chemoattractants. Signaling via PI3Ks regulates immune cell proliferation, survival, differentiation, chemotaxis, phagocytosis, degranulation and respiratory burst. The phosphorylation of phosphatidylinositol-4, 5-biphosphate (PIP2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP3) a critical second messenger molecule that urges cytoskeletal rearrangement and cell migration into the inflamed synovium is catalyzed by PI3K. Inflammatory cytokines that are produced by the newly arrived cells will further inflame the synovium and release proteases that damage cartilage and bone (Firestein, 2006).

Specific inhibitors to PI3K also inhibit the translocation of NF-κB into the nucleus, indicating that the activation of NF-κB was PI3K dependent. (Wu and Mohan, 2009). Therefore, inhibiting PI3K activity and its production of PIP3 might offer an innovative rationale-based therapeutic strategy for inflammation such as RA.

1.4. Animal models

The major goal of using animal models is to screen or validate the pharmacological activity of selected compounds aimed to behave as disease-modifying drugs (Jouzeau *et al*., 2000).

In our present research, we used Balb/c mice as an *in vivo* animal model to study the effect of *S. fruticosa* (*S. fruticosa*) on the secretion of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in lipopolysaccharide (LPS)-stimulated mice. The second model used was rat model for adjuvant-induced arthritis which served as proof of

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concept of our research at disease level. The adjuvant arthritic model represents a systemic inflammatory disease with bone and cartilage changes similar to those observed in RA. The common pathological features of adjuvant arthritis in rats and RA in humans are similar, including joint swelling associated with cellular and pannus invasion of the joint space and bone resorption (Ramprasath *et al*., 2006).

1.5. Herbal medicine

Throughout human history, herbal medicine has formed the basis of folk remedies for various inflammatory ailments. For example, the use of willow bark extract to reduce pain and fever was documented by a Greek physician (Hippocrates) in the 5th century B.C. The subsequent discovery of salicylic acid as a pain or fever relief active component gave rise to the first synthetic nonsteroidal anti-inflammatory drug (NSAID), aspirin, and the birth of the pharmaceutical industry (Ji *et al*., 2000).

1.5.1. *Salvia fruticosa*

The East Mediterranean sage *S. fruticosa* Mill (syn.: *S. triloba*) belongs to the family Lamiaceae, a large group of medicinal plants. *Salvia fruticosa* is a native plant of the Mediterranean which has been used in traditional medicine by many Asian and Middle Eastern countries to treat several ailments (Gali-Muhtasib, 2006).

In Jordan, the plant *S. fruticosa* is one of the most commonly used plant in folk medicine, almost getting extinct from the wild, but heavily cultivated, it is called Mirammeyeh. Decoctions and infusions of leaves and branches are used as common hot beverage for colic pains, treatment of colds and as a gargle for oral infections (Hudaib *et al*, 2008; Oran and Al-Eisawi, 1998).

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1.6. Objectives of Study

1.6.1. General objectives

There is no comprehensive *in vitro* and *in vivo* study that covered and evaluated the effects of *S. fruticosa* on the immune function parameters related to the development and progression of RA. The pharmacological treatment continues to be inadequate in preventing the progression of this disease to the stage of irreversible joint erosion and deformity. There is growing interest in the pharmacological potential of natural products, so the purpose of our study was to investigate the anti-rheumatic activity of *S. fruticosa* in rational-based models.

1.6.2. Specific objectives

In the present study we investigated the effects of methanolic crude extract of *S. fruticosa* in different cell types and molecules *in vitro* and *in vivo* animal models, more specifically we aimed to test:

1) Effects of *S. fruticosa* on the *in vitro* production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in:

   I. Mouse macrophage cell line RAW 264.7 cells.

   II. Mouse peritoneal macrophages.

   III. Mouse bone marrow-derived macrophages (BMDM).

   IV. Mouse splenocytes.

2) Assessment of cytotoxic effect of *S. fruticosa*;

   I. Mouse macrophage cell line RAW 264.7 cells.

   II. Mouse peritoneal macrophages.

   III. Mouse bone marrow-derived macrophages (BMDM).

   IV. Mouse splenocytes.

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3) Effects of *S. fruticosa* on cell-mediated immune response, represented by *in vitro* mixed lymphocyte reaction (MLR).
4) Effects of *S. fruticosa* on the *in vivo* secretion of pro-inflammatory cytokines in Balb/c mice.
5) Effects of *S. fruticosa* on PI3K activity *in vitro* by measuring Phosphatidylinositol(3,4,5)- triphosphate (PIP3) level in RAW 264.7 cells.
6) Effects of *S. fruticosa* on adjuvant-induced arthritis in rats as a model resemble human RA.

1.6.3. Significance of the study

I. The plant material used in this study *S. fruticosa* is widely used in Jordan and the neighboring countries for long time. (Oran and Al-Eisawi, 1998).

II. The availability of this plant in our region makes it also a cheap source for treating chronic inflammatory diseases such as RA.

III. To our knowledge, it is expected that this study is the first study, at least in our region that:

- Explore the effects of *S. fruticosa* intervention on a signal transduction pathway (PI3K) involved in development and progression of RA.
- Evaluate the effects of treatment with *S. fruticosa* on radiological changes in bone, cartilage, joint space and soft tissue in adjuvant-induced arthritis model.
- Use bone marrow derived macrophages as an *in vitro* cellular model to study the effects of *S. fruticosa* on pro-inflammatory cytokines.

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2. Literature review

2.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is the most common inflammatory arthritis and is a major cause of disability, RA is a symmetric polyarticular arthritis that primarily affects the joints of the hands, feet and knees. In addition to inflammation in the synovium, which is the joint lining, the aggressive front of tissue called pannus invades and destroys local articular structures. Various cell types such as macrophages, T cells and B cells infiltrate the synovium resulted in hyperplasia of the synovium (Firestein, 2003; Smolen *et al*., 1995).

Also, RA is a chronic autoimmune inflammatory disease characterized by prominent angiogenesis in synovial biopsies from the earliest stages of disease development. Synovium becomes markedly hyperplastic and locally invasive at the interface of cartilage and bone causing progressive destruction mediated by cytokine-induced degradative enzymes, such as the matrix metalloproteinases (Tylor and Feldmann, 2004).

Women over 40 years old are affected three times more often than men. The etiology is unknown. However, it is thought that interplay between genetic factors, sex hormones, and possibly an infectious agent or another immune-activating agent initiates an autoimmune pathogenic mechanism. (Smolen *et al*., 1995; Taylor and Feldmann, 2004).

2.2. Cellular mechanisms of RA

2.2.1. Macrophages

The innate immune response, which provides the first phase of protection against infection, requires macrophages to recognize and respond to pathogen-associated

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molecular patterns (PAMPs) as well as host-derived factors. Such mechanisms of signal integration allow fine-tuning of the host immune response, and ensure that the resulting immune response is appropriate to the immunological challenge. The importance of macrophage responses is highlighted by disease states in which macrophage function is deregulated; as inappropriate or prolonged macrophage activation is largely responsible for the pathology of acute (e.g. septic shock) and chronic (rheumatoid arthritis, inflammatory bowel disease and chronic obstructive pulmonary disease) inflammatory conditions (Schroder *et al*., 2006).

Macrophages are the first line of defense against pathogens and in response to the microenvironment, become differentially activated and play a pivotal role in promoting inflammation and joint destruction in RA. Macrophages accumulate in the synovial membrane and at the cartilage-pannus junction. The radiographic outcome and disease progression in patients with RA are correlated with the number of macrophages in the synovial lining and sublining layers (Mulherin *et al*., 1996; Tak *et al*., 1997).

Several factors that are expressed in arthritic joint such as interferon-γ (IFN-γ) can activate macrophages (Hu *et al*., 2002). Interferon-γ is a pro-inflammatory cytokine that is involved in the innate and acquired immune responses. One of the most important functions of IFN-γ is to sensitize macrophages to activation by challenge with pathogen products and IFN-γ was originally named “macrophage-activating factor “ (Schroder *et al*., 2004).

Macrophage activation can also result from direct cell-cell contact with T cells because both cell types are adjacent to each other in large numbers in the synovial membrane (McInnes *et al*., 1997).

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Macrophages and synovial fibroblasts are also in close contact with each other in the synovial membrane. Direct cell-cell interaction can occur through the ligation of CD97 on macrophages by CD55 on synovial fibroblasts (Hamann *et al*., 1999).

High levels of cytokines and chemokines, such as IL-1β, Il-6, IL-8, TNF-α, GM-CSF, MIP-1α, and monocyte chemoattractant protein-1 (MCP-1) are produced by activated macrophages in RA synovial tissue (Pope and Ma, 2005).

Tissue and cytokine microenvironment induce a heterogeneous macrophage population *in vivo* in the disease stage, which displays an appropriate inflammatory phenotype (Gordon and Varin, 2009).

Two major macrophage populations are characterized *in vitro*. Classically activated or type I macrophages, induced in particular by IFN-γ, display a pro-inflammatory profile, whereas, alternatively activated or type II macrophages (AAMQ), induced by Th2 cytokines, express anti-inflammatory and tissue repair properties (Dalton, *et al*., 1993; Gordon, 2003).

The presence of AAMQ is associated with the expression of matrix proteins such as arginase 1 which hydrolyzes L-arginine to urea and L-ornithine which is used to produce polyamines and proline to promote cell growth and collagen production (Gratchev *et al*., 2001; Louis *et al*., 1999; Modolell *et al*., 1995; Munder *et al*., 1999).

Although AAMQ are claimed to have a protective role during inflammation, its role is more complex and have many harmful outcomes in many diseases and possibly less beneficial than supposed as demonstrated by several studies (Jeney *et al*., 2002; Ponomarev *et al*., 2007).

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2.2.2. Lymphocytes

2.2.2.1. T cells

The role T cells in the pathogenesis of RA is supported by several lines of evidence. First, there is a large number of $CD4^+$ T cells infiltrating into the synovial tissue of patients with RA (Matsuoka *et al.*, 1991). Second, the receptor activator of NF-κB ligand (RANKL) from activated $CD4^+$ T cells in the arthritic joints exerts a pivotal role in bone destruction (Kong *et al.*, 1999). Third, RA is associated with a particular major histocompatibility complex (MHC) class II antigen, HLA-DR4, which apparently presents antigenic peptides to T cells. Also, $CD4^+$ T cells are involved in the stimulation of non-T effector cells to produce inflammatory cytokines such as TNF-α and IL-1 (Choy and Panayi, 2001).

2.2.2.2. The Th1/Th2 balance

Two T cell subsets have been well characterized. One of these subsets produces IFN-γ but no IL-4 T helper cells (Th1) and the other produces IL-4 but no IFN-γ (Th2). Rheumatoid arthritis is characterized by a marked shift towards the Th1 phenotype, which is described as pro-inflammatory, with overproduction of IFN-γ and inadequate production of Th2 cytokines such as IL-4 and IL-13 (Boissier *et al.*, 2008).

2.2.2.3. The Treg/Th17 balance

The role of the imbalance of Th1/Th2 in RA was refined recently by the identification of T17 and T-regulatory (Treg) lymphocyte subsets (Boissier *et al.*, 2009). Th17 lymphocytes produce IL-17, a cytokine associated with chronic

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inflammatory disease (and known as IL-17A) in the current nomenclature. The effect of Th17 cells is directly counteracted by Th2 cells, as IL-4 is a powerful inhibitor of Th17 differentiation. Th17 cells usually produce no IFN-γ, but exceptions exist. IL-17 induces the expression by fibroblast of IL-1, IL-6, TNF-α, inducible NO synthase, metalloproteases and chemokines (Steinman, 2010).

2.2.2.4. B cells

B cells have been shown to participate in chronic rheumatoid synovitis. They undergo antigen-dependent clonal expansion, affinity, maturation and differentiation into plasma cells (Kim and Berek, 2000).

Several experiments have demonstrated that B cells are involved in pathogenesis of RA. When the K/B mouse was crossed with non-obese diabetic (NOD) mouse, the resulting hybrid mouse (K/B_xN) developed very severe arthritis with a pathological typical of RA (Kouskoff *et al*., 1996).

Importantly, mice that received serum from the K/B_xN mice developed marked arthritis. Further experiments demonstrated that the arthriogenic component in the serum was anti-glucose-6-phosphate isomerase (anti-GPI) antibody produced in these animals (Matsumoto *et al*., 1999).

Another line of evidence is that B cell-deficient mice do not develop type II collagen-induced arthritis (Svensson *et al*., 1998).

Also in humans, selective B cell depletion using rituximab (an anti-CD20 monoclonal antibody) significantly improved the clinical course of RA (De Vita *et al*., 2002).

Finally, activation of B cells plays a crucial roles in the synthesis of rheumatoid factor (RF) a well recognized prognostic factor for aggressive RA, and the

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rheumatoid synovial membrane contains an abundance of B cells with RF specifity (Zhang and Bridges, 2001).

2.2.3. Neutrophils

Neutrophils are the most abundant leukocytes in human peripheral blood. They exert an important role in acute inflammation as one of the first cells to be recruited to the site of inflammation. Because neutrophils are not usually found in inflamed synovial membrane and pannus tissue, the possible role of neutrophils in RA has not received as much experimental investigation as other leukocytes such as macrophages, T cells and B cells. Activated neutrophils might release molecules such as IL-8 and complement C5a in the synovial fluid that serve as chemoattractant to recruit other leukocytes from blood to synovial tissue to worsen arthritis (Pillinger and Abramson, 1995).

In the K/B_xN mouse RA model, neutrophils have been shown to have a crucial role in initiating and maintaining inflammatory processes in the joint because mice were completely resistant to disease caused by K/B_xN serum after depletion of neutrophils (Wipke and Allen, 2001).

2.2.4. Synovial fibroblasts

Normal synovial tissue consists of two anatomically distinct layers: a surface epithelial layer (synovial lining), and an underlying layer, including macrophage-like synoviocytes (MLS) and fibroblast-like synovial cells (FLS), also called synovial fibroblasts. Synovial hyperplasia is the hallmark of RA, FLS hyperplasia has been shown to proceed the accumulation of inflammatory cells, suggesting a major role for FLS which mediate inflammation and autoimmunity through a wide

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range of mechanisms, they respond to, and themselves produce, inflammatory mediators including IL-1, IL-6, IL-8 and TNF-α.

They are also important mediators in initiation and development of arthritis. The abundance of FLS at the cartilage or pannus junction is consistent with a role for FLS in marginal cartilage erosion. The ability of FLS to erode cartilage in RA is associated largely with the secretion of matrix metalloproteinases (MMPs) which are expressed at low levels by unstimulated FLS, but are induced by IL-1 and TNF-α.

In the RA joint, FLS are one of two cell types (the other being activated T cells) responsible for production of RANKL. Thus, FLS participate in bone erosion by the activation of osteoclasts (Gravallese *et al*., 2000; Qu *et al*., 1994).

2.3. Molecular basis of RA

2.3.1. Cytokines

Cytokines are proteins secreted by the cells of innate and adaptive immunity that mediate many of the functions of these cells. Cytokines are produced in response to microbes and other antigens, and different cytokines stimulate diverse responses of cells involved in immunity and inflammation. In the activation phase of adaptive immune responses, cytokines stimulate the growth and differentiation of lymphocytes, and in the effector phase of innate and adaptive immunity, they activate different effector cells to eliminate microbes and other antigens. Cytokines also stimulate the development of hematopoietic cells. In clinical medicine, cytokines are important as therapeutic agents and as targets for specific antagonists in numerous immune and inflammatory diseases (Abbas and Lichtman, 2003).

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Cytokines have been classified as lymphokines, interleukins, and chemokines, based on their presumed function, cell of secretion, or target of action.

The term interleukin was initially used by researchers for those cytokines whose presumed targets are principally leukocytes, and the term chemokine refers to a specific class of cytokines that mediates chemoattraction between cells, and those produced by lymphocytes were called lymphokines. Cytokines are important in the regulation of the immune system, not only controlling growth and differentiation, but providing a messaging network through which different components of the immune system can interact. Over the past decade, the number of identified cytokines has expended enormously, the interleukins alone now number over 30 members. Following the recent publication of the human genome sequence, the number of known cytokines will surely increase dramatically. Given the importance of cytokines in the immune system, many of these molecules are being investigated as potential therapeutic targets, and this field of study can significantly expand in the future (Balkwill, 2000).

Although cytokines are structurally diverse, they share several properties (Abbas and Lichman, 2003):

- Cytokine secretion is a brief, self-limited event
- The actions of cytokines are often pleiotropic and redundant
- Cytokines often influence the synthesis and actions of other cytokines
- Cytokine actions may be local and systemic
- Cytokines initiate their actions by binding to specific membrane receptors on target cells
- External signals regulate the expression of cytokine receptors and thus the responsiveness of cells to cytokines

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- The cellular responses to cytokines are tightly regulated and feedback inhibitory mechanisms exist to turn down these responses.

  Cytokines include both pro-inflammatory and inflammatory peptides. The main primary pro-inflammatory cytokines include: TNF-α, IL-1, IL-6, IL-8 and IL-17.

The main anti-inflammatory cytokines include IL-10, IL-4, IL-13 and transforming growth factor beta (TGF-β).

2.3.1.1.Tumor necrosis factor-α (TNF-α)

Tumor necrosis factor α (TNF-α) is a vital pro-inflammatory cytokine involved in inflammation, immunity and cellular organization, it has emerged as the major pro-inflammatory cytokine in RA pathogenesis (Marotte and Miossec, 2010).

It was first isolated from the serum of mice infected with *Bacillus Calmette-Guerin* treated with endotoxin, and was shown to replicate the ability of endotoxin to induce haemorrhagic tumor necrosis (Carswell *et al*., 1975).

In the 1980_s, TNF-α was also characterized as cachectin for its role in wasting-first identified in rabbits infected with *Trypanosoma brucei*, and as T cell differentiation factor (Beutler *et al*., 1985; Takeda *et al*., 1986).

Cloning of the TNF-α gene in 1984 led to a decade of clinical experimentation, culminating in a license from the European Medicine Evaluation Agency for its use as a locoregional treatment for inoperable sarcoma (Lejeune *et al*., 1998).

In parallel with the emerging clinical data for TNF-α therapy in malignant disease, increasing evidence attributed a role for TNF-α in RA, inflammatory bowel disease, diabetes, sepsis, and several infections including HIV (Locksley *et al*., 2001).

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Although TNF-α is capable of initiating a tumor apoptotic response (with or without an immune-mediated mechanism), actually, these pathways are frequently deactivated within tumor cells. In some circumstances, TNF-α can provide survival signal for the cancer cell and hence, it has been referred to as a tumor promoting factor (Balkwill, 2002).

Tumor necrosis factor α is mainly produced by macrophages, monocytes, fibroblasts, and T cells, but B cells, natural killer cells (NK) and neutrophils are also minor producers of this cytokine. It is produced as a 26 KDa precursor molecule (pro TNF-α) that is membrane bound and is secreted after cleavage by TNF-α converting enzyme to yield the 17 KDa soluble mature molecule. It readily forms an active 52 KDa TNF-α trimer, which is the main extracellular form of TNF-α (Szlosarek and Balkwill, 2003; Feldmann *et al*., 1996).

Two distinct TNF receptors (TNFRs) are known: the 55 KDa and the 75 KDa receptors, also known as the p55 and p75 TNFRs. Both these molecules are transmembrane proteins, present on most cell types. The p55 and p75 TNFRs seem to be differentially regulated and transmit distinct cellular responses. Whereas the p55 TNFR is noninducible, the p75 TNFR can be induced by various stimuli. Upregulation of the p75 TNFR in macrophages can occur in response to LPS stimulation without change in the level of p55 TNFR. Both these receptors can be cleaved to yield soluble TNF receptors (sTNFR), which bind to TNF-α and prevent it from reaching its cell associated receptors. Soluble versions of both p55 and p75 receptors have been identified *in vivo*. These soluble receptors reach particularly high levels in synovial fluid from patients with RA, suggesting that (sTNFR) molecules might play a regulatory role in normal physiological responses by binding excess extracellular TNF-α (Brennan *et al*., 1995; Engelmann *et al*., 1990; Vandenabeele *et al*., 1995).

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Tumor necrosis factor α has many pro-inflammatory effects on various cell types. It is a potent activator of macrophage and inducer of IL-1 formation (Turner *et al.*, 1989). It is a growth factor for both B and T cells and a powerful activator of endothelial cells, including IL-1 release (Nawroth *et al.*, 1986) and induces the production of prostaglandin E_2, collagenase and type I and III collagens from synovial cells (Dayer and Fenner, 1992). It also plays a crucial and initiating role in inflammatory arthritis (Feldmann *et al.*, 1996). TNF-α transgenic mice can spontaneously develop polyarthritis and inta-articular injection of TNF-α can directly induce arthritis (Keffer *et al.*, 1991).

The TNF-α knockout mice are resistant to the development of arthritis (Henderson and Pettipher, 1989). Anti-TNF-α treatment potently inhibits arthritis (Feldmann and Maini, 2003).

Furthermore, experiments using RA synovial membrane cell cultures indicate that TNF-α occupies a dominant position in a cytokine hierarchy and regulates synthesis of other cytokines, Several published studies confirm that the long-term use of biologic agents targeting TNF-α lead to sustained improvements in symptoms and signs of RA (Taylor and Feldmann, 2004).

2.3.1.2. Interleukin-1 (IL-1)

Interleukin-1 is a major pro-inflammatory cytokine and its role in the inflammatory response has been well established. It has been implicated in the pathogenesis of several chronic diseases, including RA. IL-1 exists in two forms: IL-1α and IL-1β. Each form is the product of two separate genes, but is related to each other structurally at three-dimension level. Although monocytes and macrophages are the main source of IL-1α and IL-1β, these cytokines can be produced by several other cell types, including

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endothelial cells, fibroblasts and T cells (Molto and Olive, 2010; Deng and Lenardo, 2006; Dinarello, 1992).

Most IL-1α remains intracellular in its precursor form and it is believed to function as an autocrine messenger, but also there is evidence that a small part of this precursor is transported to the cell surface and associated with the cell membrane. It has been postulated that it might act as a paracrine messenger to adjacent cells and is primarily acts as a regulator of intracellular events and mediator of local inflammation. In contrast, near all IL-1β is released from the cell into the extracellular space and circulation (Dinarrelo and Wolff, 1993).

Neither IL-1β mRNA nor IL-1β protein are constitutively expressed by monocytes, macrophages or dendritic cells. Production and release of IL-1β are induced by a wide variety of stimuli, which can be divided into damage-associated molecular pattern molecules (DAMPs) and PAMPs. Invading microorganisms are recognized through PAMPs, such as LPS, lipoteichoic acid or flagellin. DAMPs such as ATP, uric acid crystals or S100 proteins, are endogenous ligands released by damaged or dying cells. Recognition of PAMPs and DAMPs is achieved by the activation of toll-like receptors (TLRs) or cystolic nucleotide binding and oligomerization domain (nod)-like receptors (NLRs) (Lotze *et al*., 2007).

Following the production of pro-IL-1β, a second signal is required to stimulate rapid and efficient processing of IL-1β and its subsequent release. Investigations on LPS-stimulated monocytes and macrophages revealed that in the absence of a second stimulus, cells release very slowly only small amounts of mature IL-1β into the extracellular milieu. Rapid processing and release of IL-1β can be initiated by PAMPs or DAMPs and is regulated by the active caspase-1, also known as interleukin-1-

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converting enzyme (ICE). Activation of caspase-1 is promoted by the NLR family members (Eder, 2009).

The mechanisms of IL-1β secretion are poorly understood. In general, secretion of proteins can occur through classical or non-classical secretory pathways. The majority of secretory proteins, such as the pro-inflammatory cytokines IL-6 and TNF-α are externalized through the classical pathway. These proteins are first translocated across the endoplasmic reticulum membrane, and are then further transported through the Golgi apparatus to the plasma membrane, either directly or via secretory granules.

Interleukin-1β belongs to the group of leaderless proteins which lack a conventional hydrophobic signal sequence. These proteins are released through a non-classical secretory pathway independent of endoplasmic reticulum and Golgi apparatus. (Halban and Irminger, 1994).

Different models of a non-classical secretory pathway have been postulated to explain the mechanisms of IL-1β release; in particular, secretion of IL-1β has been suggested to occur: (i) via exocytosis of secretory lysosomes, (ii) by shedding of plasma membrane microvesicles, (iii) via exocytosis of exosomes or (iv) by export through the plasma membrane using specialized transporters. In addition, leakage of cell contents from lysed cells has been suggested as a possible mechanism of IL-1β release (Qu *et al*., 2007).

There are two different IL-1 receptors (IL-1Rs), namely IL-1R1 and IL-1R1 and IL-1 II. Binding of IL-1α or IL-1β to IL-1R1 produces activation, subsequent intracellular transduction and cellular responses, whereas binding to IL-1R II does not tranduce signal. IL-1R II seems to be a decoy receptor, acting as a buffer to compensate excessive concentrations of IL-1 (Dinarello, 1996).

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The pro-inflammatory effects of IL-1 are countered by the endogenous IL-1 receptor antagonist (IL-1Ra) by functioning as a competitive inhibitor for the binding of IL-1. Secreted IL-1Ra is an inducible gene in most cells, but intracellular IL-1Ra is expressed constitutively in keratinocytes and intestinal epithelial cells. IL-1Ra represents the first naturally occurring cytokine or hormone-like molecule acting as a specific receptor antagonist. The systemic and synovial fluid concentrations of IL-1 are raised in RA, and they correlate with disease severity and histological features. IL-1Ra levels are also increased in many patients with RA, but they may not be sufficient for keeping IL-1 activity in balance (Chikanza *et al*., 1995).

Interleukin-1 has many biological functions that are of importance in inflammatory diseases. It stimulates production of acute phase proteins by the liver, including IL-6, fibrinogen, complement components, and various clotting factors. Augmented IL-1 production has been found in patients with viral, bacterial, fungal and parasitic infections. Increased IL-1β levels have also been related to atherosclerosis, type 2 diabetes and various autoimmune disorders, such as rheumatoid arthritis, multiple sclerosis and Crohn's disease. Neurodegenerative diseases that are accompanied by inflammatory process, such as Alzheimer's disease or Parkinson's disease, have also been characterized by augmented IL-1β production (Allan *et al*., 2005; Church *et al*., 2008).

The proliferation of fibroblasts in RA is enhanced by IL-1, leading to formation of pannus and triggers the destruction of cartilage, bone and periarticular tissues by activating synovial fibroblasts and chondrocytes. Also, elevated levels of IL-1 are found in the early stages of experimental and clinical arthritis. Intra-articular injection of IL-1 into rabbit knee joints induces arthritis (Keffer *et al*., 1991). Deficiency of IL-1Ra gene in mice causes autoimmunity and arthritis indicating a vital function in inflammatory

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response homeostasis in the body. Anakinra (human recombinant IL-1Ra) has been shown to decrease the severity and incidence of arthritis in several animal models and clinical trials (Furst, 2004).

Furthermore, Th17 differentiation in humans is promoted by IL-1β and the current hypothesis is that RA is caused by a Th1/Th17 imbalance with predominance of Th17 (Boissier *et al*, 2008).

2.3.1.3. Interleukin-6 (IL-6)

Researches conducted to look for soluble factors produced by T cells, capable of inducing B cell proliferation and differentiation led to the identification of IL-6. Initially, 36 different names were used for IL-6, reflecting the broad array of effects induced by this cytokine. These names included B cell stimulating factor-2 (BCSF-2), interferon-β2 (IFN-β2), hepatocyte stimulating factor (HSF), macrophage granulocyte inducer type 2 (MGI-2A), and thrombopoietin. In 1986, the complementary DNA of the cytokine was cloned and studied. Several groups then suggested using a single name, IL-6 to avoid confusion. The name IL-6 was finally chosen at a conference held in New York in December 1998 (Assier *et al*., 2010).

Interleukin-6 is a cytokine that can facilitate autoimmune phenomena, amplify acute inflammation and promote the evolution into a chronic inflammatory state. In addition, it is a major promoter of bone resorption in pathological conditions (Fonseca *et al*., 2009).

A wide variety of cell types, including T and B cells, macrophages, activated monocytes, mast cells, neutrophils, eosinophils, glial cells, fibroblasts, chondrocytes, adipocytes, osteoblasts and endothelial cells are involved in IL-6 production (Kishimoto, 1989).

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The IL-6 receptor is a heterodimer composed of a specific 80-KDa chain (IL-6Rα) and of a 130-KDa transmembrane chain (gp130) responsible for signal transduction.

Whereas gp130 is expressed ubiquitously, IL-6Rα is expressed only by hepatocytes, phagocytes, and a few lymphocytes. IL-6 binds first to the α chain, which facilitates the interaction between IL-6Rα and gp130. Dimerization of these three molecules (IL-6/IL-6Rα/gp130) allows signal transduction via gp130, which activates an intracellular pathway of the Janus kinases (JAKs) then the signal transducer and activator of transcription 3 (STAT3) and NF-κB leading to the expression of genes that are critically involved in inflammation and lymphocyte migration (Mcloughlin *et al*., 2005).

The soluble form of IL-6Rα (IL-6Rαs) is capable of enabling IL-6 activities. After IL-6Rαs binds IL-6 in solution, the complex can interact with gp130, which is found at the surface of all cells in the body. Thus, although the expression of IL-6Rα is restricted, the existence of the soluble form allows cells that do not express IL-6Rα to respond to IL-6. This mechanism is known trans-signaling (Assier *et al*., 2010).

The production of IL-6 is deregulated in RA. IL-6 acts on multiple cell targets, which explain that it has been found involved in both the local and the systemic manifestations of RA. IL-6 found in large amounts in the synovial fluid and serum of patients with RA. In the joint, the main source of IL-6 may be the fibroblastic synovial cell, although lymphocytes and activated macrophages also produce IL-6 (Yoshizaki *et al*., 1998). It can act locally on the vascular endothelial cells. Activation of these cells by IL-6 leads to the production of chemokines and adhesion molecules, which promote leukocyte recruitment to the site of inflammation. The differentiation of osteoclast and fibroblastic synovial cell activation require IL-6, thus, contributing to the development of the synovial pannus and breakdown of the bone and cartilage. The role for IL-6 in the

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systemic manifestations of RA is illustrated by the effects on the hepatocytes by initiating and modifying acute-phase protein production (Assier *et al*., 2010).

The involvement of IL-6 in the immunological manifestations of RA is illustrated by its effects on B cells, it stimulate B cell proliferation and differentiation to antibody-producing plasma cells, which may cause hypergammaglobulinemia and the production of autoantibodies such as RF and antibodies to cyclic citrullinated peptides (ACPA) in the serum and joints. These antibodies are disease markers that may be present very early, well before the onset of the clinical symptoms (Jacobi and Dorner, 2010).

Interleukin-6 is also involved in the orientation and differentiation of T cell. Once activated, naïve T cells differentiate at the periphery to regulatory T cells (Treg) or effector T cells (Teff). The inflammatory process increases the differentiation of naïve T cells to Th17. In mice, naïve T cells differentiate to Treg cells at the expense of Th17 cells in the presence of TGF-β alone, whereas they differentiate to Th17 cells in the presence of both TGF-β and IL-6. In humans, the conditions conducive lead to naïve T cell differentiation to Th17 cells are different from those in mice and consists of IL-6 combined with IL-1β and IL-23 (Acosta-Rodriguez *et al*., 2007).

2.3.1.4. Receptor activator of nuclear factor (RANK and RANKL)

Osteoclasts arise from mesenchymal stem cell and differentiate from hematopoietic monocyte or macrophage precursors. Osteoclasts are the principal effector cell of bone destruction in inflammatory arthritis because of their ability to degrade bone. Imbalances between osteoclasts and osteoblasts activities can cause a greater the amount of bone to be removed during the bone resorption phase than the amount of bone laid down during the bone formation phase. This process is a major cause of bone destruction (Boyle *et al*., 2003).

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The TNF-family molecule RANKL ligand and its receptor RANK are key regulators of bone remodeling and essential for the development and activation of osteoclasts. The knockout mice for RANK or RANKL display severe osteopetrosis and lack mature osteoclasts. In RA, T cells and macrophages are dominant disease-causing cells and RANKL is mainly produced by activated T cells, monocytes, macrophages and osteoblasts (Deng and Lenardo, 2006). There are large quantities of RANK and RANKL expression and mature osteoclasts at disease sites in inflammatory arthritis (Deng *et al*., 2005).

Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL and competes with RANK for RANKL binding. Inhibition of RANKL via OPG had no effect on the severity of inflammation. However, OPG treatment completely prevented the reduction in mineral bone density in the inflamed joints of adjuvant-induced arthritis in a dose-dependent manner. Bone destruction in untreated arthritic rats correlated with a dramatic increase in osteoclast numbers, whereas, OPG treatment reversed the accumulation of osteoclasts. So, it is clear that RANKL is chief mediator of joint destruction and bone loss in adjuvant arthritis. Importantly, untreated rats experienced severe crippling arthritis. By contrast, rats treated with OPG at the onset of disease-analogous to a patient consulting a doctor at the onset of joint swelling did not show the severe clinical signs of crippling arthritis (Deng and Lenardo, 2006).

2.3.1.5. Nuclear Factor (NF-κB)

The nuclear factor-κB (NF-κB) family of transcription factors is among the most intensively studied in vertebrate biology. Since their discovery in the 1980_s, they have been shown to be involved in many different pathways, including inflammation, cell survival, proliferation and differentiation. Activation of the NF-κB is normally pro-

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inflammatory. However, they are also anti-apoptotic and a delicate balance between the two functions must be tightly regulated. The NF-κB family consists of five members; p50, p100, p65(also known as RelA), RelB and c-Rel. These NF-κB subunits form homodimers and heterodimers to produce NF-κB transcription factors. The most common activating form present in synovial cells and activated macrophages of patients with RA is a heterodimer of p50 and p65. In unstimulated cells, NF-κB transcription factors are found in the cytosol, rendered inactive by an inhibitor of κB (IκB) molecule. Activation of the IκB kinase (IKK) complex targets IκB for degradation, thus releasing NF-κB (Simmonds and Foxwell, 2008).

In particular, TNF-α, is the prime inflammatory mediator and also induces apoptosis. The genes encoding TNF-α and many of the other factors such as cytokines(IL-1β and IL-6), chemokines (IL-8 and MCP-1), MMPs (MMP-1 and MMP-3) and metabolic proteins (Cox-1, Cox-2 and iNOS) are now known to be under the control of NF-κB transcription factors, suggesting that NF-κB could be one of the master regulators of inflammatory cytokines production in RA (May and Ghosh, 1998).

The presence of activated NF-κB transcription factors has been demonstrated in cultured synovial fibroblasts and human arthritic joints (Yamasaki *et al*., 2001).

New techniques such as *in vivo* imaging has also been used to demonstrate the activity of NF-κB in a mouse model that mimicked RA-like chronic inflammation. By placing the luciferase gene under the control of NF-κB, incresde luminescence was observed in the joints of live mice (Carlsen *et al*., 2002).

Furthermore, the above findings are supported by a study that investigated experimentally induced arthritis in mice that carried knockouts of the genes for the NF-κB family members p50 or c-Rel. The two experimental models used were collagen-

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induced arthritis (CIA), and an acute /destructive model induced by methylated bovine serum albumin (BSA) and IL-1. Lack of c-Rel had no influence on the acute model and whilst reducing the incidence of CIA, did not prevent a severe immunohistopatholoy in affected joints. In addition, c-Rel could not be found in the nuclei of cells explanted from the arthritic joints of wild-type mice, suggesting that this subunit of NF-κB is of limited importance in RA. In contrast, lack of p50 caused a complete loss of a humoral response, severely imbedded T cell proliferation conferred resistance to both forms of arthritis, which clearly demonstrates a central role of p50 (presumably p50/p65 heterodimers) in the inflammation that underlies RA (Campbell *et al*., 2000).

2.4. Signal transduction pathways in RA

2.4.1. The phosphoinositide 3-kinases (PI3Ks)

The phosphoinositide 3-kinases (PI3Ks) comprise a family of enzymes that utilize both lipid and protein kinase activity to regulate numerous intracellular transduction pathways, which in turn coordinate a range of downstream cellular processes, including cell growth, differentiation, survival, proliferation and migration. Based on sequence homology and lipid substrate specifity, the PI3K family is divided into three classes (I, II and III). The class I PI3Ks have received the most attention from the scientific community and are further broken down into two subclasses, IA and IB, both of which catalyze the phosphorylation of phosphatidylinositol (4,5)-biphosphate (PIP2) to produce the signaling molecule phosphatidylinositol (3,4,5)-triphosphate (PIP3) (Ameriks and Venable, 2009).

The holoenzyme consist of a regulatory subunit (designated p50, p55, p85, or p101) and a catalytic subunit (designated p110α, p110β, p110γ, or p110δ) that are essential for their recruitment to the plasma membrane and subsequent generation of the key lipid

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second messenger PIP3. Activation of this signaling pathway can occur through two distinct receptor types, receptor tyrosin kinases (RTK) or G-protein coupled receptors (GPCRs), each of which utilizes specific p110 isoforms. For instance, the RTK is thought to primarily activate α, β and δ isoforms of p110 (designated class IA), while the GPCR only p110γ (designated class IB). However, this classification may not be so straightforward, as many reports demonstrate that PI3Kγ in macrophages can be activated indirectly through the RTK and that PI3Kδ contributes to PIP3 production in neutrophils in response to GPCR activation by lipid mediators of inflammation or bacterial products. Such observations would suggest that the function of these two classes of PI3K may overlap in particular subsets of leukocytes and that the activation of these signaling pathways may not be restricted to a particular type of receptor (Randis *et al*., 2008).

Whereas PI3Kα and PI3Kβ are ubiquitously expressed, PI3Kδ and PI3Kγ are mainly restricted to leukocytes and represent key modulators of innate and adaptive immune responses. The best characterized PI3K effector molecule is the protein kinase PKB or Akt (PKB/Akt), which accumulates at sites of PI3K activation through a direct interaction between pleckstrin-homology domain (PH) and the PIP3, thus triggering numerous intracellular signaling pathways (Ghigo and Hirsch, 2008).

Signal transduction pathways are activated by cytokines and other factors such as viral or bacterial proteins, heat and ultraviolet radiation. These stimuli act on receptors that are coupled to the signal transduction pathway via a submembraneous apparatus, causing activation of transcription factors that control the production of cytokines, proteases, growth factors and many other compounds involved in the inflammatory process. Inhibition of signal transduction pathways would be expected to abolish both cell activation by cytokines or other stimuli and production of new molecules of pro-

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inflammatory cytokines. Cytokines activate many signal transduction pathways which engage in a high level of crosstalk. Signal transduction pathways closely involved in inflammation include the JAK-STAT pathway, PI3K pathway, MAPK, and NF-κB.

The membrane receptor undergoes a conformational change as a result of ligand binding which results in phosphorylation of the receptor itself or of a receptor-associated enzyme. The first phosphorylation activates a cascade of enzymes called protein kinases, which in turn activate transcription factors (Morel and Berenbaum, 2004).

Tumor necrosis factor-α in RA is thought to be produced mainly by macrophages, and this production is regulated by NF-κB. In T cells NF-κB activity is required for normal T cell receptor (TCR) signaling and differentiation of naïve T cells into Th1 phenotype. Also NF-κB plays a crucial role in the regulation of B cell maturation and is required for normal osteoclast development and function (Tas *et al*., 2005).

Both PI3K and PKB did exhibit activation in response to LPS in RAW 264.7 cells and that this activation was sensitive to the fungal metabolite wortmannin which was initially isolated from *Penicillium wortmanni* and was subsequently shown to be a specific inhibitor of PI3K , and the synthetic compound LY294002 which was designed as PI3K inhibitor based on the naturally occurring flavonoid derivative quercetin (Salh *et al*., 1998).

A large number of patent specifications have been published recently, which describe new PI3K inhibitors, claiming the use of these novel compounds in the treatment of a wide range of PI3K-mediated diseases. Targeting signal transduction molecules, especially kinases is a hard task. First, these enzymes contribute to a vast array of cellular processes essential for survival and are often widely distributed in many tissues. Second, the target enzyme is often structurally similar to others and many such enzymes

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share substrates such as ATP. Whereas, the PI3Kα and PI3Kβ isoforms are ubiquitous and are activated by many extracellular stimuli and cellular stresses, the expression of PI3Kγ and PI3Kδ is far more restricted, it is found primarily in hematopoietic cells. Moreover, the stimuli that activate PI3Kγ are comparatively few and include chemokines and other chemoattractant proteins such as C5a fragment of complement, that signal through GPCRs. So, PI3Kγ is an attractive therapeutic target for immune and inflammatory disease because it is expressed mainly in leukocytes and regulates the transport of cells into inflammatory sites after activation of chemokine receptors. Because many chemokines and chemoattractant receptors signal through PI3Kγ, one can potentially suppress overlapping pathways at once (Firestein, 2006).

Severalstudies of a mouse model of RA support concept that mice lacking PI3Kγ gene, as compared with wild-type mice, had significantly less synovial inflammation and joint destruction, on the basis of both the clinical extent of arthritis and a histological evaluation of the joint. This particular model relies on the ability of systemically administrated antibodies against collagen to bind cartilage and activate complement locally. The release of chemotactic factor such as C5a in the joint after complement fixation and activation of Fc receptors on resident cells recruits blood leukocytes to the joint and leads to synovitis. Camps and colleagues developed novel PI3Kγ inhibitors with a degree of selectivity, as determined by *in vitro* assays using monocytes and macrophages. One compound completely arrested disease progression in the standard CIA model (Camps *et al*., 2005).

The activity of PI3K is crucial for leukocyte function, but the roles of the four receptor-activated isoforms are unclear. Mice lacking GPCRs PI3Kγ were viable and had fully differentiated neutrophils and macrophages. Chemoattractant-stimulated $PI3K^{-/-}\gamma$ neutrophils did not produce PIP3, did not activate PKB, and displayed impaired

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respiratory burst and motility. Peritoneal PI3Kγ^{-null} macrophages showed a reduced migration toward a wide range of chemotactic stimuli and a severely defective accumulation in a septic peritonitis model, demonstrating that PI3Kγ is a crucial signaling molecule required for macrophage accumulation in inflammation (Hirsch *et al.*, 2000).

2.5. Therapies for RA

Intense investigation of the cause of RA has uncovered many of the integral biochemical, cellular and molecular pathological components and pathways of this disease, developing and marketing of new and novel therapeutics that target several seminal components of RA.

Many of today's disease- modifying antirheumatic drugs (DMARs) have entered rheumatology owing to clinical intuition rather than theoretical reasoning and their mechanism of action remain unknown. However, there are many different presumed mechanisms of DMARs actions such as suppression of TNF-α and IL-1, inhibition of macrophage activation, induction of apoptosis of the inflammatory cells, reducing the number of T cells and as an antifolate activity (Capell *et al*, 2007). For example, the gold complexes were first used as tuberculostatics and later in bacterial endocarditis;, it is reported that the patient's joint pain was ameliorated by the injections of aurothioglucose led to this compound being tried out in RA.

The antimalarias were derived from the bark of the Peruvian cinchona tree and were originally used against certain fevers. Quinine was used in systemic lupus erythematosus (SLE) as early as 1894. In the early 1950s, quinacrine was tried in a large SLE material, and it was noted that some patients with associated RA also improved. This observation led to the use of antimalarias in RA, which is still widespread in some

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countries. Another early antiarthritic compound, sulfasalazine, entered rheumatology in the 1930s, being synthesized as a combination of an (antibacterial) sulfonamide moiety and an (anti-inflammatory) salicylic acid derivative, because it was at that time widely believed that RA was an infectious disease (Bondeson, 1997).

2.5.1. Anti-inflammatories, analgesics and the cyclooxygenase-2 inhibitors

Aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs), (e.g. Advil®, Voltaren®, Proxen®) have, for many years, been a front-line therapy for treating the pain and inflammation in RA. An unfortunate consequence of NSAID therapy is the common occurrence of gastrointestinal irritation. Although analgesics (e.g.Panadol®, Endocet®) are free of this gastrointestinal liability, and do relieve RA-associated pain, they do not affect joint inflammation. Sometimes, physicians treat RA patients with a combination of an NSAID and a gastroprotectant, such as a proton pump inhibitor, histamine receptor antagonist or cytotec. The pharmaceutical industry has tried to discover, develop and market an efficient NSAID with little or no gastrointestinal undesirable effects. This has led to the development of the cyclooxygenase-2 (COX-2) inhibitor, a new class of NSAID that is represented by Vioxx®, celebrex® and Bextra®. In 2004, Vioxx® was voluntary withdrawn from the market by Merck because it has been shown to increase the risk of heart attacks and stroke in some individuals participating in a colon polyps trial after 18 months of therapy. Furthermore, in 2005 Pfizer withdrew its COX-2 inhibitor Bextra® as ordered by Food and Drug Administration (FDA or USFDA).

Although Pfizer was permitted to continue marketing another COX-2 inhibitor, Celebrex®, FDA ordered Pfizer to place a “ black box” warning in the package insert

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addressing the fact that patient's taking Celebrex® (as with Vioxx® and Bextra®) were at increased risk of suffering from heart attacks and/or stroke. The FDA also stipulated that serious warnings must be provided for all drugs in the NSAID class (Smith, 2005).

2.5.2. Disease-modifying antirheumatic drugs (DMARDs)

Whereas DMARDs such as methotrexate, sulfasalazine, hydrochloroquine, leflunomide and azathioprine were until now administrated as a second-line RA therapy, they have recently become a popular choice for many rheumatologists.

Methotrexate is currently the first treatment option for many physicians because of its efficacy and relative safety. It has been demonstrated that DMARDs suppress the clinical manifestations of RA, slow the radiographic progression of joint erosions (Smith, 2005).

2.5.3. Corticosteroids

Corticosteroids (glucocorticoids) have been used as therapies for RA. When administrated as a low-dose and short-duration therapy, corticosteroids (e.g. prednisone) exert remarkable anti-inflammatory effects, suppressing the clinical signs, symptoms and early-stage disability of RA. Definitive data have also shown these drugs display DMARD-like activity in retarding the progression of joint erosions. However, their broad spectrum of side effects prevent their use by many rheumatologists (Bijlsma *et al.*, 2002).

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2.6. Biological agents

2.6.1. Cytokine-targeted strategies for the treatment of RA

The targeting of pro-inflammatory cytokines, especially TNF-α and IL-1, has become a strategic basis for developing therapies to treat RA. Approaches that have been investigated include soluble TNF receptors, soluble IL-1 receptors, IL-1 receptor antagonists and monoclonal antibodies to TNF-α.

Two of these agents, both of which neutralize TNF-α, have received FDA approval for the treatment of the disease. Enbrel® is a bioengineered fusion protein of the p75 soluble TNF receptor, and Remicade® is a chimeric monoclonal antibody to TNF-α (Carteron, 2000).

2.6.2. Soluble TNF receptors

Both the p55 and p75 receptors participate in signal transduction pathways. The p75-based molecule, Enbrel® consists of two soluble p75 sTNFR joined to the Fc portion of a human immunoglobulin G1 (IgG1) molecule. Analogously, the p55-based molecule, lenercept is composed of two p55 sTNFR linked to IgG1-Fc.

Improvement with Enbrel® was extremely rapid, with decreases in measures of disease activity noted within two weeks of initiation of therapy, but symptoms returned after therapy discontinuation (Carteron, 2000).

2.6.3. Soluble IL-1 receptors

Two types of IL-1 receptors have been detected on the cell surface. Only the type I receptor appears to mediate signal transduction. The type II receptor appears to act as a decoy. Soluble forms of IL-1 receptor have been identified in plasma. A soluble form of

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the type I IL-1 receptor, recombinant human IL-1R1 (rHuIL-1R1), has been tested in phase I trials of patients with active RA, but the efficacy of this agent has not proven particularly promising (Drevlow *et al*., 1996).

In addition to binding IL-1, soluble IL-1 receptors (sIL-1Rs) also bind the IL-1 receptor antagonist (IL-1Ra). This interaction might complicate the therapeutic use of sIL-1Rs, particularly the type I sIL-1R, because the affinity of this molecule for IL-1Ra is higher than its affinity for IL-1α or IL-1β. The type II sIL-1R, on the other hand, binds to IL-1β more tightly than to the IL-1Ra. This molecule might therefore have more therapeutic potential as an IL-1 neutralizer than does type I sIL-1R (Arend *et al*., 1994).

2.6.4. Monoclonal antibodies

Another strategy for inhibiting cytokines is to neutralize cytokines with specific monoclonal antibodies. Three monoclonal antibodies directed against TNF-α have been developed for clinical use: a chimeric monoclonal antibody (infliximab), a humanized monoclonal antibody (CDP571) and a fully human monoclonal antibody (D2E7).

The therapeutic use of antibodies to treat RA have drawbacks that include their potential to cause cell lysis through complement fixation, and loss of efficacy due to neutralizing antibody formation such as the development of anti-infliximab antibodies in patients with RA treated with infliximab (Carteron, 2000).

2.6.5. Interleukin-6 and IL-6R monoclonal antibodies

Because of its involvement in several autoimmune diseases such as RA, IL-6 is a promising treatment target. In early experiments, monoclonal antibodies to IL-6 were developed. This strategy failed in clinical trials in humans, as the antibodies formed immune complexes with IL-6. Another strategy involved targeting the receptor instead

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of the cytokine itself. A humanized monoclonal antibody to IL-6Rα was generated (tocilizumab/Actemra®/RoActemra®, Roche/Chugai).

Tocilizumab blocks the effect of IL-6 by limiting its ability to bind to its receptors. Clinical trials in patients with RA established that tocilizumab reduce the severity of symptoms and returned the C-reactive protein (CRP) levels to normal (Assier *et al.*, 2010).

2.7. Targeting intracellular signaling pathways

Alterations in the transcription and translation of specific proteins can occur due extracellular signals that are transduced intracellularly by multiple pathways. The end result of some of these signaling pathways is the production of proteins including cytokines and MMPs, which are implicated in the pathogenesis of RA (Piecyk and Anderson, 2001).

Intracellular signaling pathways have been implicated in the various stages of the inflammation and joint destruction process that characterizes RA. They can be blocked by specific inhibitors which are already used to treat various malignancies. Several of the fundamental components of the receptor-coupled signal transduction pathways that are closely linked to the pathogenesis of RA have become the subject of significant biomedical investigation. The signal travels in the cell via a sequence of kinase-induced activating phosphorylations or phosphatase-induced inhibiting dephosphorylations.

Inhibition of signal transduction pathways might abolish not only cell activation induced by cytokines or other stimuli, but also the production of new molecules of pro-inflammatory cytokines.

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Inhibiting the kinases that induce phosphorylation blocks intracellular signal transduction. Thus, kinase inhibitors may hold promise as treatments targeting intracellular signaling pathways (Wendling *et al*., 2010).

Abnormal functioning, differentiation and/or activation of T cells, B cells and myeloid cells have been documented in various autoimmune diseases. More recent studies have also demonstrated irregular activation of various signaling axes including various MAPK, PI3K/Akt, NF-κB and JAK/STAT molecules in these cells. Among these, one molecular pathway that appears to be particularly attractive for therapeutic targeting is the PI3K/Akt/mTOR (mammalian target of rapamycin) axis (Wu and Mohan, 2009).

2.8. Herbal medicine

Although drugs such as NSAID and DMARDs, have a reasonably good effect on symptomatic relief of arthritis, the current treatment still not satisfactory to modify fundamental pathologic processes responsible for the chronic inflammation. Cytokine-based therapies have been used for the treatment of RA and found to be useful in preventing progression of chronic arthritis in groups of patients. However, the later therapies are based on the use of exogenous proteins (such as antibodies), which are costly and have the possibility of causing an immune response against the exogenous protein and need for systemic injection (Barsante *et al*., 2005).

Considering the potential adverse effects of these drugs, as well as their limited ability to provide long-term remission, new conventional drugs and complementary alternative medicine (CAM) products, including herbs, are continuously being demanded.

Herbal products are receiving increasing public interest, and the herbal treatment is now the most popular CAM therapy.

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2.8.1. Chinese herbal medicine

Chinese herbal medicine, a major type in traditional Chinese medicine (TCM) and practiced for thousands of years in China and other Asian countries, is used for treating arthritis and related disorders.

Herbal formulations are the common form of administration in Chinese herbal practice, and herbal formulas are well documented in ancient and modern literature. According to Chinese herbal theory, interactions among the different herbs in a formula exert a synergistic effect and neutralize potential toxicity and side effects of the individual constituents. However, there is as yet lack of accurate and rigorous scientific evaluation of such formulas (Zhang *et al*., 2009).

The traditional Chinese herbal drug Dan-Shen (Tan-shen, *S. miltiorrkiza* Bge.) is described to have sedative, antimicrobial, antispasmodic, anti-inflammatory and antioxidant properties. Tan-shen is mentioned in Chinese Pharmacopoeia as a drug that treats problems associated with heart and circulatory system, insomnia and as a drug used in the treatment of acute arthritic pain in patients with rheumatism (Xiao, 1989).

Historically, Chinese culture has relied heavily on herbal treatment of many illnesses. Traditional herbal preparations still account for 30-50% of the total medicinal consumption in China. Written records document the use of Chinese herbal medicine over 3000 years ago (Wieser *et al*., 2007).

Using traditional medicinal knowledge in drug discovery seems so promising that recently even large pharmaceutical companies have begun to show interest. For example, Novartis, a Swiss drug giant, has invested 100 million US dollars in a new research and development center in Shanghai that will bring together modern biomedical research and millennia old medicinal concepts. It is also Novartis- in collaboration with the world health organization (WHO) - that produces and distributes

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Coartem® (Riamet®) a front line antimalarial drug. Coartem® is derived from quinghausu (artemisinin) a sequiterpene peroxide first isolated from the TCM herb *Artemisia annua* L. by Chinese scientists in 1971.

In 2006, more than 62 million Coartem treatment courses delivered to more than 30 countries. So it is clear that scientific examination of historic works can be the base for the " rediscovery " of long forgotten remedies and a source of information for a more focused screening for new leads (Adams *et al*., 2009).

2.8.2. Medicinal plants in the Mediterranean area

The Mediterranean region, despite its location in a temperate zone far from the diversity hotspots popularized by the media, it is one of the areas with the greatest diversity on the planet, and thus it is considered that it should be maintained as a conservation sanctuary. Some 10% of the world's higher plants can be found in this area, which represents only 1.6% of the Earth's surface. Around 25,000 species are found in the region, and a very high percentage of these are endemic. The current pharmacopeia of Western countries has its origin in the works of classical authors, such as Theophrastus or Dioscorides, which means that an abundant wealth of plants and medicinal uses in the Mediterranean basin. This ancient tradition of using medicinal plants has not been reflected in an ethnobotanical study line until relatively recent times, when researchers have realized the urgent need to collect this knowledge, which represents a rich heritage both for the exploration of new resources as well as for constituting a irreplaceable part of the traditional culture of Mediterranean peoples. Nevertheless, many local remedies used throughout the Middle East region have never been properly explored, researched, evaluated or exploited, compared for example, Chinese medicine, which is farther away from Europe in theory (Gonzalez-Tejero *et al*., 2008).

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2.8.3. Arab herbal medicine

For many centuries after the fall of the Roman Empire, the Arabic world was the center of scientific medical knowledge. Texts from Greece and Rome were translated into Arabic and studied by Islamic scholars. They developed and refined Hippocrates theories and Islamic physicians began to use the regulation of diet, exercise and the prescription of medicinal herbs in the treatment of their patients. Arabs in the Baghdad region were the first in history to separate medicine from pharmacological sciences. It is very important to note the fact that the first models of professional pharmacy were found among the Arab population. The first drugstores in the world were established in the Arab world (Baghdad, 754). The forms used in that period are still used in the therapy and some formulations of drugs can be found in pharmacopeias even today. Experts in the pharmaceutical sciences undertook the extraction and preparation of remedies. Pharmacologists and ethnopharmacologists started to search for different ingredients and extracts to be used as remedies, and studied the chemical properties of materials used in disease management. In contrast to the historical importance of Arab medicine, current research into the different modalities of CAM in our region and the know-how of modern Arab herbalists are limited (Azaizeh *et al*., 2006).

2.8.4. Medicinal herbs in Jordan

Jordan is considered a meeting point for three continents: Asia, Africa and Europe. This unique location has led to diversity in climate, geology and topography.

Geographically, Jordan is divided into four different zones: the Mediterranean, the Irano-Turanian, the Saharo-Arabian and the Sudanian. Within these diverse zones, there are a total of 13 different vegetation types each with many different floral and faunal elements (Aburjai, 2007; Al-Esawi, 1996).

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Although Jordan is relatively a small country, it is characterized by great variation in wild plants. Around 2500 plant species (of which 100 species (2.5%) are listed as endemic) were recorded. The floral species in Jordan also include medicinal and herbal species. From these plants, 485 species from 99 different families are categorized as medicinal plants, which are widely distributed all over the country (Oran and Al-Esawi, 1998).

Like other countries in the region, Jordan is composed of two different societies: one rural and the other urban. Both of them depend upon the rich traditional heritage. Folk medicine is widely practiced by the inhabitants of the remote areas or the nomads who generally inhabit the desert and some areas of the steppe and the uplands. The reliance on herbal medicine and the uncontrolled collection of medicinal plants might cause the disappearance of some medicinal herbs growing in the area and will add more plants to the list of the endangered plant species (Afifi and Abu-Irmaileh, 2000).

2.9. The sage *Salvia*

Salvia species (sage) belong to the Lamiaceae family (formerly, Labiatae). The genus name *Salvia* L. is derived from the Latin *salvare* meaning “to heal or to be safe and unharmed” referring to the medicinal properties of some of the species. It has been used for centuries, especially by the Chinese to promote longevity and in Roman ceremonies as a sacred herb. The positive benefits of *Salvia officinalis* (common sage) to health are reputed throughout Ancient Romans times and the Middle Ages. A quote such as “Why should a man die whilst sage grows in his garden? “Epitomizes the impact of this sage on that society at the time. *Salvia africana-lutea* was used by early Europeans settlers in the Western Cape as an infusion to treat colds.

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Before the discovery of antibiotics, it was frequently prepared as a component of herbal tea mixture, to treat tuberculosis and chronic bronchitis (Kamatou *et al*., 2008).

Salvia is the largest genus of the Lamiaceae family, *Salvia* L., is represented by over 900 species and is widely distributed in various regions of the world, namely, the Mediterranean areas, South Africa, Central and South America, and Southeast Asia. The plants are typically 30-150 cm tall, herbaceous or suffruticose, and perennial, rarely biennial, or annual, with attractive flowers in various colors.

Salvia species have been used since ancient times for more than sixty different ailments ranging from aches to epilepsy, and mainly to treat colds, bronchitis, tuberculosis, hemorrhage, gingivitis, and menstrual disorders. The main secondary metabolite constituents of *Salvia* species are terpenoids and flavonoids. The areal parts of these plants contain flavonoids, triterpenoids, and monoterpenes, particularly in the flowers and leaves, while diterpenoids are found mostly in the roots (Topu, 2006).

2.9.1. *Salvia fruticosa*

Salvia fruticosa Miller (syn: *S. triloba*) is another well-known *Salvia*. *Salvia fruticosa* (*S. fruticosa*) is native to the eastern Mediterranean, including southern Italy. It also occurs on the Canary Islands and in North Africa. Due to its wide variation in leaf shape, there has been a great deal of taxonomic confusion over the years, with many of the leaf variations of *S. fruticosa* being named as distinct species. These include *S. lobryana*, *S. libanotica*, and *S. cypria*. Adding to the confusion over the name, the plant has also been called *S. triloba*, as named by Carl Linnaeus in 1781, until it was discovered that it was the same as the plant named by Philip Miller in 1768, with the earlier name receiving preference according to plant naming conventions (Clebsch and Barner, 2003; Topu, 2006).

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Salvia fruticosa is the most popular medicinal herb in Jordan, Palestine and Lebanon (Abu-Rmaileh and Afifi, 2000; Ali-Shatayeh *et al*., 2000; Gali-Muhtasib and Affara, 2000).

Twenty species of *Salvia* grow wildly in Jordan (Oran and Al-Esawi, 1998). The leaves of the plant are boiled and used as a herbal tea for treatment of bloating, gastric disorders, abdominal pain, oral infections, gum and tooth pains, headaches, cough, influenza and cold, feminine sterility, skin disorders, nervous conditions, asthma, rheumatism, and diabetes (Abu-Rmaileh and Afifi, 2000; Ali-Shtayeh *et al*., 2000; Gali-Muhtasib *et al*., 2000; Oran and Al-Esawi, 1998; Salah and Jager, 2005).

In Jordan, the plant is also used for the treatment of ulcer pains and indigestion. In Turkey, it is used for gallbladder and kidney stones and these effects are claimed to be due to the activity of its essential oils such as α-Pinene, β-Pinene, Cineole, α-Thujone, β-Thujone, Linalool, Linalyl acetate and Borneol (Gali-Muhtasib *et al*., 2000).

Phytochemicals derived from plants and plant extracts have been widely studied for their anti-inflammatory, antioxidant, upregulation of detoxifying enzymes and suppression or activation of different signal transduction pathways (Neergheen *et al*., 2010).

As inflammation is a host response to infections, traumas, and autoimmune conditions, aimed to aid the healing process and trigger an immune response against pathogens but, when deregulated, it can lead to inflammatory diseases as in the case of rheumatoid arthritis.

Pharmacological control of these diseases is managed using steroidal and NSAIDs. However, due to their adverse effects, particularly after prolonged use, there are still unmet medical needs in the treatment of such diseases. A promising alternative to these drugs are products of plant origin.

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Because of the curative potential of the genus *Salvia*, it can be regarded as a promising group of plants with anti-inflammatory properties. And since different members of the genus are traditionally used to treat inflammations and an actual antiphlogistic effect has been demonstrated for several species and/or their constituents. In addition, *salvia* species show high diversity in bioactive constituents mainly flavonoids and terpenoids (Sosa *et al*., 2009).

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3. Materials and Methods

3.1. Herbal sample

Arial parts of *S. fruticosa* were purchased from the local market and the plant was identified by Prof. Dawud Al-Eisawi, Department of Biological Sciences, and a voucher specimen has been deposited at the herbarium, University of Jordan.

The herbal material was air-dried for five days, then minced into small pieces weighing 432 grams (gm) and grinded until powdered into fine powder.

3.2. Extraction

The plant material was extracted by using Soxhlet apparatus, methanol used as the solvent, the extraction time was 24 hours and then evaporated under vacuum using rota vapor from Heidolph Instruments (Germany), the evaporation of the solvent resulted in crude extract paste-like material weighing 62.5 gm.

3.3. Animals

Female Balb/c mice were obtained from the animal house, Faculty of Medicine, University of Jordan. Mice were inbred at the animal house. The average weight of the mice used was 23-28 gm and 6-8 weeks old, they were kept in plastic cages at conventional conditions of humidity, light and temperature randomized into 4 groups and housed 6 mice/cage for the pro-inflammatory cytokines secretion *in vivo* experiment. They were fed with laboratory diet and tap water *ad libitum* and acclimatized for one week prior the experiment.

Male *Sprague Dawley* rats also were inbred at the animal house. The average weight of rats was between 230-270 gm and 8-10 weeks old. They were kept in plastic cages in

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the same conditions of mice, randomized into 3 groups and housed 6 rats/cage for the adjuvant-induced arthritis experiment.

3.4. Culture media

Raw 264.7 cells from American type culture collection (ATCC), mice splenocytes and peritoneal macrophages were cultured in RPMI-1640 medium from Gibco (USA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) from Gibco (USA), 100 U/ml penicillin from Sigma (USA), 100 µg/ml streptomycin from Sigma (USA) and 2 mM L-glutamine from Sigma (USA), 50 mM-mercaptoethanol (2ME), 10 mM HEPES buffer (N-2-hydroxyethylpiperezin-N\-(2-ethanesulfonic acid) from Sigma (USA). To maintain logarithmic growth, Raw 264.7 cells were passaged every 2-3 days at humidified atmosphere in 37 °C incubator with 5% CO_2 until they were used (Uchya *et al*, 1999).

3.5. Chemicals, reagents, kits, disposables and instruments

Lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli*) serotype 0111:B4 was purchased from Sigma (USA), phosphate-buffered saline (PBS) from Gibco (USA), bovine serum albumin (BSA) from Sigma (USA), Trypsin ethylenediaminetetraacetic acid (Trypsin EDTA) from Lonza (Belgium), 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium (MTT) from Sigma (USA), Dimethylsulfoxide $(CH3)_2$ SO (DMSO) from Merck (USA), 2,2́-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate from Sigma (USA), complete Freund's adjuvant (CFA), product number F5881 from Sigma (USA), each ml of CFA contains 1 mg of heat-killed and dried *Mycobacterium tuberculosis* (strain H37Ra, ATCC 25177), 0.85 ml paraffin oil and 0.15 ml of mannide monooleate, single and multichannel micropipettes from Gilson

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(France), tissue culture flasks and 96-well plates for ELISA assays from Nunc (Denmark), sterile disposable tubes 15 and 50 ml from Greiner (Germany), syringe-driven filters 0.22 µm from Jet Biofil (Canada), murine ELISA kits for measurement of TNF-α, IL-1β and IL-6 from Peprotech (UK), PIP3 mass ELISA kit product number K-2500 from Echelon (Salt lake city, UT), ELISA plate reader from Biotek (USA), digital plethysmometer from Panlab Harvard apparatus (Spain), cell scrapers from Nunc (Denmark), concanavalin A (Con A) from Sigma (USA) extracted from *Canavalia ensiformis*, mitomycin C (from *Streptomyces caespitosus*) product number M4287 from Sigma (USA), Hank's balanced salt solution (HBSS) from Lonza (Belgium), Trypan blue(0.4%) from Gibco (USA), Red blood cells (RBCs) lysing buffer contain 8.3 g/L ammonium chloride (NH_4Cl) in 0.01 M Tris- HCl buffer, product number R7757 from Sigma (USA), Eagle's modified essential medium (EMEM) from Gibco (USA), Tween-20 from Sigma (USA) and macrophage colony-stimulating factor (M-CSF) product number M9170 from Sigma (USA).

3.6. Isolation and preparation of murine splenocytes, peritoneal macrophages and bone marrow-derived macrophages

3.6.1. Removal of spleen from mice

Mice were sacrificed by cervical dislocation and then immersed in a beaker filled with 70% ethanol to reduce the possibility of contamination, the animal placed on its back on clean, dry, absorbent filter paper under the laminar flow. A midline incision was made with a scissors and the skin was retracted above the neck and below the thighs by pulling it with gloved fingers. The left side of the animal was exposed for spleen removal, the incision was made at the left of the peritoneal wall with surgical scissors and the spleen was grasped as much as possible with curved forceps and gently pulled

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free from the peritoneum, tearing the connective tissue behind the spleen, then the spleen was immediately immersed in RPMI-1640 medium in a sterile plate. The same procedure was done for the rats.

3.6.2. Preparation of splenocytes single cell suspension

The single cell suspension of splenocytes was made by teasing the spleen between the frosted edges of two sterile microscopic slides, then the mixture of the medium and cells in the plate was aspirated into a sterile conical tube and centrifuged at 1020 rpm for 10 minutes (min) at 4°C in a swing bucket refrigerated centrifuge (Sigma, Germany). After centrifugation, the supernatant was discarded and the pellet was suspended in 5 ml of RBCs lysing buffer and incubated for 3-5 min at room temperature. The lysed RBCs were washed 3 times in 10 ml RPMI-1640 medium. After the last wash, the supernatant was decanted and the pellet was resuspended in RPMI-1640 medium and the viable cells count was done by using trypan exclusion test.

3.6.3. Trypan exclusion test for cell viability

Trypan blue is a vital dye. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable.

In this test, one part of the cell suspension is mixed with one parte of 0.4% trypan blue (e.g. 100 µl+100 µl, dilution 1:2) the mixing was done by a micropipette and the mixture is kept for 3 min at room temperature, then a drop of trypan blue/cell mixture was applied to a hemacytometer. Cells were counted within 5 min of mixing with trypan blue, as longer incubation period will lead to cell death and reduced viability counts.

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The unstained (viable) and staine (nonviable) cells in the hemacytometer were counted by using a binocular light microscope. To obtain the total number of viable cells per ml of aliquot, the total number of viable cells was multiplied by 2 (the dilution factor). To obtain the total number of cells per ml of aliquot, the total number of viable and nonviable cells was added and multiplied by 2 and the percentage of viable cells was calculated according to the following formula:

$$\text{Viable cells (\%)} = \frac{\text{Total number of viable cells per ml of aliquot}}{\text{Total number of cells per ml of aliquot}} \times 100$$

(Freshney, 1987; Strober, 1997).

3.6.4. Isolation and preparation of peritoneal macrophages

To collect the resident peritoneal cells, 10 female Balb/c mice were sacrificed by cervical dislocation and then immersed in a beaker filled with 70% ethanol. The animal placed on its back on clean, absorbent filter paper under the laminar flow. A midline incision was made with a sterile scissors and the abdominal skin was retracted with a sterile forceps to expose the intact peritoneal wall. A 5-ml syringe with 19-G needle was filled with 11.7% sucrose chilled solution, with the beveled end of the needle facing up, the needle was inserted through peritoneal wall at the midline and the sucrose solution was injected into the mouse. Another syringe (with the needle beveled end down) was inserted into the peritoneum and raised slightly to cause tenting of the peritoneal wall and starting to withdraw the peritoneal fluid slowly, then the needle was removed from the syringe and the peritoneal fluid was dispensed and collected in a 50-ml sterile conical polypropylene centrifuge tube on ice. Then, the peritoneal lavage fluid was centrifuged for 10 min, 1020 rpm and 4 °C. After centrifugation, the supernatant was discarded and the cells pellet was resuspended in RPMI-1640 medium, mixed

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thoroughly and cell count was done with trypan blue on a hemacytometer as mentioned previously (Donovan and Brown, 1995).

3.6.5. Isolation of mice bone marrow-derived macrophages (BMDM)

In order to obtain BMDM, 20 female Balb/c mice were sacrificed by cervical dislocation, and then the mice were immersed in a beaker filled with 70% ethanol. The skin was peeled from the top of each hind leg and downed over the foot with a sterile scissors and forceps. The foot was cutted off with the skin and discarded. The hind legs were cutted off and placed in a sterile dish containing sterile PBS under the laminar flow. The muscle from legs was removed by holding the end of the bone with forceps and using scissors to push the muscle downward away from forceps. The leg bones between joints were severed. A 10-ml syringed attached to 26-G needle was filled with sterile PBS, and then the needle was inserted into the bone marrow cavity of femur and tibia. The bone cavity was flushed with PBS until the bone cavity appeared white, then the wash mixture (gelatinous bone marrow and PBS) was collected and transferred into 50-ml sterile conical polypropylene centrifuge tube on ice. Then, the tube was centrifuged for 10 min, 1600 rpm at room temperature. After centrifugation, the supernatant was discarded. The resulted pellet was resuspended in EMEM supplemented with 10% FBS, HEPES buffer (10 mM)), L-glutamine (2 mM), Gentamicin (50 µg/ml), penicillin (100 U/ml), streptomycin sulfate (100 mg/ml) and also containing M-CSF (10 ng/ml), and then cultivated in a standard tissue culture flask (25 cm^2) in 5% CO_2 incubator with a humidified atmosphere at 37 °C for at least 24 hours. After that, the non-adherent cells were transferred to another tissue culture flask (75 cm^2). The cells were incubated for 4 days in 20 ml of EMEM and followed by 3 days incubation in 30 ml of EMEM with 10% FBS and M-CSF (10 ng/ml). The cells

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were harvested using Trypsin- EDTA solution and the viable cell count was done by trypan blue exclusion test as mentioned previously (Cunnick *et al*., 2006; Donovan and Brown, 1995).

3.7. Preparation of plant extract solution and concentrations

For *in vitro* experiments (splenocytes, peritoneal macrophages, BMDM, RAW 264.7 cells and mixed lymphocyte reaction (MLR)), 10 mg of *S. fruticosa* crude extract was dissolved in 10 ml RPMI-1640 medium in sterile conical polypropylene centrifuge tube to obtain a stock plant extract solution of 1000 µg/ ml concentration. Then, this solution was sonicated in ultrasound sonicator from BANDELIN Electronic (Germany) for 60 min, after sonication it was centrifuged for 10 min, 4000 rpm at room temperature to remove the solid precipitate which was weighed 1 mg, then the solution was sterilized by using a syringe-derived filter 0.22 µm under the laminar flow and then serial dilutions of the stock were made in RPMI-1640 medium to obtain the desired extract concentrations to be used in the experiments. The concentrations were 250, 125, 62.5 and 31.25 µg/ml (Ibrahim and Aqel, 2010; Kaileh *et al*., 2007).

For *in vivo* experiments (pro-inflammatory cytokines in mice and adjuvant-induced arthritis in rats), the concentration prepared to be 2 g/kg (2 mg extract/ gram body weight) dissolved in sterile PBS and sonicated, centrifuged and sterilized same as preparation for *in vitro* experiments above (Ramirez *et al*, 2007).

3.8. Cell viability and proliferation assay using MTT

The cytotoxic effect of *S. fruticosa* crude methanolic extract on the cells being involved in the experiments was evaluated by the conventional MTT assay (the mitochondria-dependent reduction of MTT to formazan), 20 µl of the MTT solution (5 mg/ml in PBS,

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pH 7.4) was added to the corresponding wells in triplicate in a 96-well plate and incubated for 4 hours in 5% CO_2 incubator in humidified atmosphere at 37 °C. After incubation, the supernatant was aspirated by 1-ml syringe and discarded, then 200 µl of DMSO was added to each well and mixed thoroughly with a multichannel pipette to dissolve the crystals, the optical density (O.D) then was measured by a spectrophotometer plate reader with a test wavelength of 570 nm and reference wavelength of 630 nm (Seo *et al*., 2001; Tao *et al*., 2008).

3.9. Mixed lymphocyte reaction (MLR) *in vitro*

Three female Balb/c mice were killed by cervical dislocation and one female Albino rat was killed by chloroform inhalation in a closed jar. Splenocyte single cell suspension and count were prepared as previously mentioned in materials and methods (section 3.6.2). Mice splenocytes were assigned as the responder (R) cells, while the rat splenocytes were the stimulator cells (S). Stimulator splenocytes were inactivated by treatment with mitomycin C (50 µg/ml) and incubated at 37 °C in 5% CO_2 incubator with humidified atmosphere for 45 min, followed by 3 washes with RPMI-1640 medium. Mixed lymphocyte cultures were established by combining 0.4 x 10^6 /well responder cells with 0.6 x 10^6 /well stimulator cells. Each responder and stimulator cell population was plated in triplicate in 96-well sterile round bottom plate as a control for each, also mixed lymphocyte culture was plated in triplicate in a total volume of 0.2 ml of RPMI-1640 medium and all the border wells of the plate were filled with 200 µl of RPMI-1640 medium to prevent evaporation in the test wells. Xenogenic mixed lymphocyte cultures were treated with different concentrations of *S. fruticosa* extract (31.25-250 µg/ml) and incubated for 5 days in a humidified 5% CO_2 incubator at 37 °C,

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and then the MTT assay was done. The responder, stimulator, mixed lymphocyte cultures and RPMI-1640 medium were added in the following combinations:

1) Responder + stimulator (R+S) + plant extract at various concentrations. The purpose of this combination is to study the effect of *S. fruticosa* on proliferation in the mixed culture.
2) Responder + stimulator (R+S) + RPMI-1640 medium. This combination was done without extract treatment, which expected to have the highest proliferation activity.
3) Responder + RPMI-1640 medium (R+M). This combination was done in order to test the spontaneous proliferation of splenocytes that neither being stimulated, nor treated and it is expected to show high proliferation activity, but not higher than the (R+S) + RPMI-1640 medium combination.
4) Stimulator + RPMI-1640 medium (S+M). The purpose of this combination is to make sure that the stimulator cells are no more capable of proliferation, because they were already inactivated by mitomycin C.

3.10. Measurement of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) production *in vitro* by ELISA and cytotoxic effect of *S. fruticosa* extract by MTT

3.10.1. Measurement of pro-inflammatory cytokines in RAW 264.7 cells

The adherent RAW 264.7 cells in the tissue culture flask were harvested by discarding all the media from the flask followed by washing w8ith PBS without Ca^{+2} and Mg^{+2} and then the cells were incubated with Trypsin-EDTA for 5-10 min,

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in 5% CO_2 in humidified atmosphere at 37 °C for detachment. After the incubation period, cells were detached by beating the plate on the bench and by adding RPMI-1640 medium to the flask with continuous and fast mixing with a sterile pipette attached to a pipette aid and collected in a sterile conical polypropylene centrifuge tube on ice, and then, centrifuged for 10 min, 1020 rpm at 4 °C. After centrifugation the supernatant was decanted and the pellet was resuspended in RPMI-1640 medium, cell count was adjusted to 2 x 10^6 /ml. 100 µl (0.2 x 10^6 cells/well) of this cell suspension was loaded in 96-well plate in triplicate for each group of cells as following:

1) Positive control group (+ve), cells were stimulated with LPS (1 µg/ml), (100 µl of cell suspension+100 µl RPMI-1640 medium) a total of 0.2 ml/well, to test the pro-inflammatory cytokines production level in LPS-stimulated RAW 264.7 cells and to determine the successful establishment of the experiment (Guo, *et al*., 2008; Hwang *et al*., 2009).
2) The test group, cells were subjected to pretreatment with *S. fruticosa* extract at various concentrations (31.25-250 µg/ml) for one hour before being stimulated with 1 µg/ml LPS, to test the effect of the plant extract on cytokines production level in these cells.
3) The extract-treated group, cells were treated with the same extract concentrations as in the test group, but without LPS stimulation, to study the effect of the plant extract on the baseline level of pro-inflammatory cytokines.
4) The normal control group, cells were only incubated with RPMI-1640 medium, without extract treatment or LPS stimulation, to study the effect of plant extract on pro-inflammatory cytokines baseline production level.

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After overnight (18 hours) incubation in 5% CO_2 incubator in a humidified atmosphere at 37 °C, the supernatant of each group was collected in sterile eppendorf tubes on ice, then aliquots of 200 µl were kept and stored at -21 °C until use within one week.

Measurement of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in each group's supernatant was done in triplicate for each cytokine as well as for the standards for each cytokine and RPMI-1640 medium under the same conditions used as the blank.

The test for cytokines was done by using murine ELISA development kits according to the manufacture instructions, briefly, as following:

100 µl of capture antibody was added to each ELISA plate well, sealed and incubated overnight at room temperature and then, the wells were aspirated and washed 4 times using 300 µl of wash buffer per well (wash buffer: 0.05% Tween-20 in PBS). After the last wash the plate was inverted to remove residual buffer and blotted on paper towel followed by addition of 300 µl block buffer to each well and incubated for at least 1 hour at room temperature (block buffer: 1% BSA in PBS). After incubation, the wells were aspirated and washed 4 times. Immediately, 100 µl of each standard concentration (0-4000 pg/ml) or sample was added to each well in triplicate and incubated at room temperature for at least 2 hours. The plate, then, aspirated and washed 4 times and 100 µl/well of detection antibody was added to all wells and incubated at room temperature for 2 hours. After that, the plate was aspirated and washed 4 times and 100 µl of Avidin-HRP conjugate was added to all wells and incubated for 30 min at room temperature. The plate was aspirated and washed 4 times and 100 µl of ABTS liquid substrate was added to all wells and incubated at room temperature for color development. The color development was monitored with an ELISA plate reader at 405 nm with wavelength correction set at 650 nm at 5-min intervals for approximately 45 min. The net optical density (O.D) 405-650 nm was used to establish the standard curve and to calculate the

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cytokine concentration for each group. This protocol was the same for TNF-α, IL-1β and IL-6. The differences were in capture antibody, standards and detection antibody for each cytokine.

3.10.1.1 Assessment of effect of *S. fruticosa* on viability of RAW 264.7 cells

RAW 264.7 cells (0.2 x 10^6 cells/well) same as the density used for cytokine secretion and overnight incubation treated with same *S. fruticosa* extract concentrations used in cytokine experiment (31.25-250 µg/ml) and a normal control group of cells, only incubated with RPMI-1640 medium without extract treatment in 5% CO_2 incubator with humidified atmosphere at 37 °C in order to test the effect of the plant extract on the viability of the extract-treated cells.

Another plate was loaded with RAW 264.7 cells (2 x 10^4 cells/well) treated with plant extract (31.25-250 µg/ml) and a normal control group of cells incubated only with RPMI-1640 medium without extract treatment in 5% CO_2 incubator with humidified atmosphere at 37 °C for 3 days to test the effect of *S. fruticosa* extract on proliferation of these cells. Both the viability and proliferation of RAW 264.7 cells were evaluated by MTT assay.

3.10.2. Measurement of pro-inflammatory cytokines in peritoneal macrophages

Peritoneal cells suspension was plated (2.0 x 10^6cells/well) in 96-well plate with RPMI-1640 medium and incubated for 2 hours in 5% CO_2 incubator with a humidified atmosphere at 37 °C. After this incubation time, the nonadherent cells were removed by

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repeated washing with PBS (Fronhofer *et al*., 2006; Jeon *et al*., 1999; Liang *et al*., 2009).

To the adherent cells in the plate, immediately, 200 µl of RPMI-1640 medium was added in the following design:

RPMI-1640 with LPS (5 µg/ml) to the positive control group.

RPMI-1640 with *S. fruticosa* extract at various concentrations (31.25-250 µg/ml) to the test group and incubated for 1 hour before the addition of LPS (5 µg/ml).

RPMI-1640 medial only, was added to the normal control group.

Then, the plate was incubated for 18 hours (overnight) in 5% CO_2 with a humidified atmosphere at 37 °C. The supernatant of each group was aspirated and processed as mentioned in section 3.10.1 of materials and methods (RAW 264.7 cells).

3.10.2.1. Assessment of cytotoxic effect of *S. fruticosa* on peritoneal macrophages

Peritoneal macrophages (2.0 x 10^6 cells/well) same as the density used for cytokine secretion and overnight incubation, treated with same *S. fruticosa* extract concentrations used in cytokine experiment (31.25-250 µg/ml) and a normal control group of cells, only incubated with RPMI-1640 medium without extract treatment in 5% CO_2 incubator with humidified atmosphere at 37 °C in order to test the effect of the plant extract on the viability of the extract-treated cells by performing MTT assay.

3.10.3. Measurement of pro-inflammatory cytokines in BMDM

The mice BMDM cells were harvested using Trypsin-EDTA solution and were planted in 96-well plate (0.5 x 10^6 cells/well) with RPMI-1640 medium in the same order of the peritoneal macrophages experiment section 3.10.2 of materials and methods and the

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experiment was conducted exactly as done with peritoneal macrophages, except that, the LPS concentration used was 1 µg/ml.

3.10.3.1. Assessment of effect of *S. fruticosa* on viability of BMDM

The mice BMDM (0.5 x 10^6 cells/well) same as the density used for cytokine secretion and overnight incubation, treated with same *S. fruticosa* extract concentrations used in cytokine experiment (31.25-250 µg/ml) and a normal control group of cells, only incubated with RPMI-1640 medium without extract treatment in 5% CO_2 incubator with humidified atmosphere at 37 °C in order to test the effect of the plant extract on the viability of the extract-treated cells by performing MTT assay.

3.10.4. Measurement of pro-inflammatory cytokines in splenocytes

Mice splenocytes were planted in 96-well plate (2 x 10^6 cells/well) with RPMI-1640 medium and assigned into 4 groups, as following,

The positive control group was stimulated with ConA (2.5 µg/ml).

The test group was pretreated with *S. fruticosa* extract (31.25-250 µg/ml) for 1 hour before being stimulated with ConA (2.5 µg/ml).

The only extract-treated group was incubated with RPMI-1640 medium containing plant extract only in the same concentrations used for the test group, to test the effect of the plant extract on the baseline level of the pro-inflammatory cytokines in unstimulated cells.

The normal control group, only incubated with RPMI-1640 medium, to test the level of pro-inflammatory cytokines in unstimulated cells.

The plate was incubated for 18 hours (overnight) in 5% CO_2 with humidified atmosphere at 37 °C. After the end of incubation, the supernatant of each group was

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collected and processed as mentioned previously for the other cell types (RAW 264.7 cells, peritoneal macrophages and BMDM).

3.10.4.1. Assessment of effect of *S. fruticosa* on viability of splenocytes

Viability of splenocytes with same loading density (2 x 10^6 cells/well), same grouping design used in cytokine experiment and same incubation period was evaluated by MTT. Another plate, was planted with splenocytes (0.2 x 10^6 cells/well) and same grouping design, but with prolonged incubation period for 3 days was performed to test the effect of *S. fruticosa* on proliferation activity of splenocytes, also this was done by using MTT assay.

3.10.5. Measurement of pro-inflammatory cytokines production level in Balb/c mice as *in vivo* model

In this experiment, a total of 24 mice were used with an average weight of 25 gm and divided into four experimental groups as following:

The positive control group was intraperitonealy (i.p) injected with 1 ml PBS containing 25 µg LPS (25 µg/ml; 1 µg LPS/gm weight) for each mouse (Mouna *et al*., 2010; Robert *et al*., 2002).

The test group was injected (i.p) with 1 ml PBS containing 50 mg *S. fruticosa* extract (2gm extract/kg weight) before 1 hour of stimulation with 25 µg/ml LPS.

The only extract-treated group was injected (i.p) with 50 mg plant extract, to test the effect of the plant extract on the pro-inflammatory cytokines secretion level in unstimulated mice.

The normal control group was injected (i.p) with the vehicle (PBS) only, to test the baseline level of pro-inflammatory cytokines in unstimulated mice.

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All groups, except the test group, were injected with 1 ml PBS before 1 hour of LPS, extract or vehicle injection.

After 2 hours from the last injection in all groups, blood was collected from mice by bleeding from the neck by a sterile surgical blade. Serum was obtained by centrifugation of the clotted blood and aliquots of 200 µl were kept frozen at – 21 °C to be used within one week in ELISA assay for TNF-α, IL-1β and IL-6.

3.10.6. Examination of PI3K activity by measuring PIP3 level in RAW 264.7 cells

The majority of PIP3 synthesized in response to extracellular stimuli is, most likely, generated by phosphorylation of PIP2. The class I PI3Ks are the only enzymes that can use PIP2 as a substrate to synthesize PIP3 (Rameh and Cantley, 1999).

This experiment was conducted by stimulating RAW 264.7 cells with LPS (1 µg/ml) for 10 min (Kang *et al*., 2003; Salh *et al*., 1998). This was the control group.

The second group was the test group, which was treated with *S. fruticosa* extract at concentrations (31.25-250 µg/ml) for 1 hour before being stimulated with 1 µg/ml LPS.

The third group was the normal control group, which was neither stimulated, nor treated, to measure the PIP3 level in unstimulated cells.

The experiment was conducted in duplicate for each group with cell density of 7 x 10^6 cells/group in 75 cm^2 tissue culture flask, incubated in 5% CO_2 incubator with a humidified atmosphere at 37 °C.

At the end of incubation period, the cells and the cellular material was precipitated by immediate addition of 4 ml of ice-cold 0.5M trichloroacetic acid (TCA), and incubated on ice for 5 min, according to the extraction protocol provided with the kit. The cells were scrapped off with cell scraper and transferred into 15 ml sterile polypropylene

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centrifuge tube on ice and the flask was rinsed with additional 4 ml 0.5M TCA and the precipitate was pelleted by centrifugation at 1500 rpm for 5 min at 4 °C. The pellet was then washed two times with 1 ml of 5% TCA 1 mM EDTA. Neutral lipids were extracted from the pellet with 1ml of methanol: chloroform 2:1 by vortexing three to four times over a 10-min period at room temperature. This extraction was repeated and the solvent supernatants were discarded. The acidic lipids were then extracted as follows: 750 µl chloroform: methanol: 12 M HCL 40: 80: 1 was added to the pellet and vortexed occasionally over a 15-min period at room temperature. A phase split was carried out by the addition of 250 µl chloroform and 450 µl 0.1 M HCL followed by centrifugation to separate the organic and aqueous phases.

The organic phase was collected into sterile polypropylene tube and dried in a vacuum drier. The pellet at this stage was just visible. The organic-phase lipids were used for PIP3 quantitation using PIP3 mass ELISA kit K-2500 from Echelon (Salt lake city, UT) according to the manufacturer′s instructions. Briefly, the lipid extract from the cultured cells was first mixed with the PIP3-specific detector protein, which was then incubated in PIP3-coated microplate for competitive binding. After several washes, the microplate was then incubated with HRP-linked secondary detector and tetramethylbenzidine (TMB) substrate for color development. To stop further color development, 2 M H_2SO_4 solution was then added. Microplates were read at 450 nm wavelength for absorbance. A series of different dilutions of PIP3 standards were used for establishing a standard curve (Costa *et al*., 2007; Gray *et al*., 2003; Huang *et al*., 2007).

3.10.7. Adjuvant-induced arthritis in rats

In this experiment, rats were randomly distributed into three groups, 6 rats/group and the experiment was conducted as following:

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The arthritic group (diseased group), rats in this group were slightly anesthetized with chloroform and injected subcutaneously at the tail base with 150 µl of a suspension of CFA. This day was considered the zero day.

The treated group, rats were injected (i.p) with *S. fruticosa* extract dose of 2 g/ kg before 1 hour of being subcutaneously injected at the tail base with 150 µl CFA in the same day (zero day).

The normal control group was only injected (i.p) with PBS.

Daily, after the zero day, the rats in the treated group were injected (i.p) with the plant extract for 20 days. The experiment period was 25 days.

Rats in the arthritic and normal group were daily injected (i.p) with PBS for 20 days in order to be exposed to the same conditions of the treated group.

Rats were inspected on alternate days for symptoms of clinical arthritis using a macroscopic scoring system by grading each paw from 0 to 4, where 0 = no erythema or swelling; 1 = slight erythema or swelling of one of the toes or fingers; 2 = erythema and swelling of more than one toe or finger; 3 = erythema and swelling of the ankle or wrist; 4 = erythema or swelling of the entire paw. The clinical score was calculated by adding the scores for four individual legs, so the highest possible arthritic index is 16.

The clinical severity of arthritis was also determined by quantitating the change in the paw volume with a plethysmometer (Donovan and Brown, 1995; Jawed *et al*., 2010; Klareskog and McDevitt, 1999).

3.10.8. Radiographic analysis

Moreover, in order to be more confident and precise that the above observations and measurements are truly related to arthritis, especially, when the clinical symptoms became very clear, radiographic analysis was done on the day 17th of the experiment.

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Lateral radiographs were obtained with the table-top technique for both hind paws after moderate anesthesia with chloroform, with an X-ray unit Hitachi XG-2012/33 (Hitachi medical corporation, Japan). Al radiographs were evaluated by a board-certified radiologist unaware of the treatment group assignment.

3.10.9. Statistical analysis

Student ́s *t* test was used for determining the statistically significant differences between values of various experimental groups. Data were expressed as means ± SD and a *p* value ≤ 0.05 was considered statistically significant.

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4. Results

4.1. Assessment of inhibitory activity of *S. fruticosa* on pro-inflammatory cytokines production by *in vitro* cellular models and on viability.

4.1.1. Effect of *S. fruticosa* on TNF-α, IL-1β and IL-6 production by RAW 264.7 cells

As shown in Fig. 1, *S. fruticosa* extract at concentrations 250, 125 and 62.5 μg/ml significantly inhibited TNF-α production by Raw 264.7 cells up to 63%, 55% and 45% respectively, in a dose-dependent manner, compared to the single LPS-stimulated cells (the positive control group). The TNF-α level reached 26650 pg/ml, then falling to 9734 pg/ml at the highest concentration of the plant extract, 250 μg/ml ($p < 0.05$). The other concentrations (125 and 62.5 μg/ml) of *S. fruticosa* also reduced TNF-α level to 12087 pg/ml and 14665 pg/ml respectively ($P < 0.05$). At the lowest concentration used (31.25 μg/ml), TNF-α also was decreased by 30%, but it was statistically not significant ($P > 0.05$). However, *S. fruticosa* did not affect TNF-α level in the absence of LPS at all concentrations used compared to the normal control group, where TNF-α level was only 517 pg/ml.

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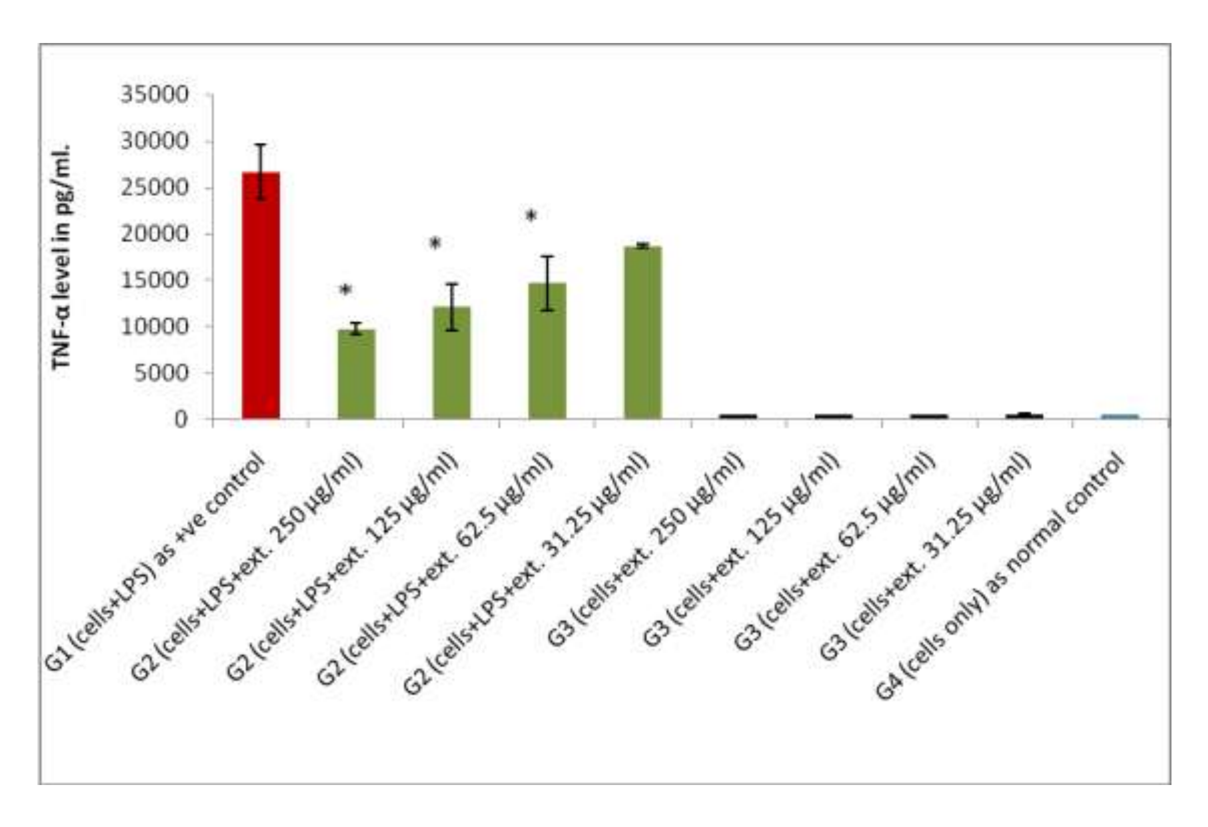


Figure 1. Effect of *S. fruticosa* on TNF-α production by RAW 264.7 cells G1: Positive control (red column); G2: Treated group with *S. fruticosa* and LPS (green columns); G3: Extract-treated group without LPS (black columns); G4: Normal group (blue column) * $p < 0.05$ compared to the positive control group.

In Fig. 2, IL-6 production by RAW 264.7 cells was strongly suppressed by *S. fruticosa* at all concentrations used (31.25-250 µg/ml), the inhibition reached up to 91% at the highest concentration 250 µg/ml and up to 73%, 46% and 43% other concentrations 125, 62.5 and 31.25 µg/ml respectively in a dose-dependent manner compared to the single LPS-stimulated cells (the positive control group) reaching 45762 pg/ml, then falling to 3681, 12179, 24436 and 25634 pg/ml respectively ($P < 0.05$).

The IL-6 level was below the detection limit of the kit (64 pg/ml) in cells treated with 250, 125 and 62.5 µg/ml plant extract only, while not affected at lowest dose 31.25 µg/ml compared to the normal group.

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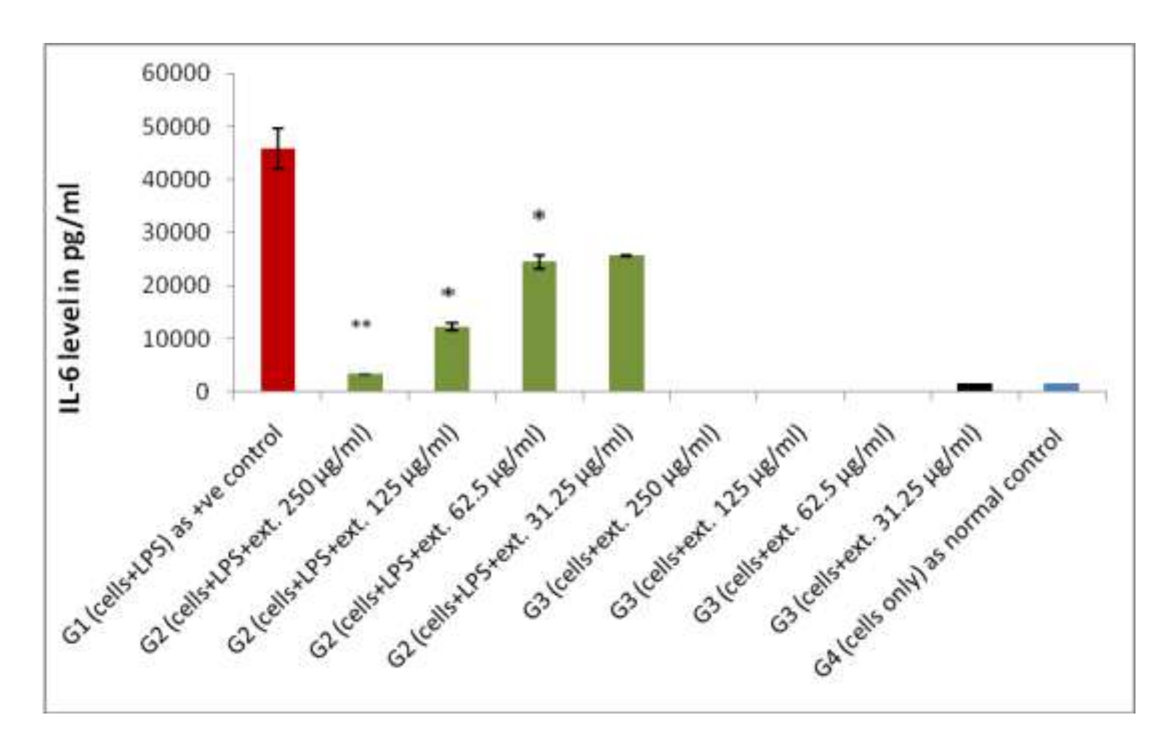


Figure 2. Effect of *S. fruticosa* on IL-6 production by RAW 264.7 cells G1: Positive control (red column); G2: Treated group with *S. fruticosa* and LPS (green columns); G3: Extract-treated group without LPS (black columns); G4: Normal group (blue column) ** $p < 0.01$, * $p < 0.05$ compared to the positive control group.

The production of IL-1β was slightly increased in the single LPS-stimulated cells (positive control) reaching only 400 pg/ml compared to the unstimulated group (normal control) reaching 213 pg/ml (Fig.3). However, this increase was statistically significant ($P < 0.01$). Also, *S. fruticosa* showed significant inhibitory effect for IL-1β level in cells treated with LPS and plant extract at all concentration used ($p < 0.01$). Also, there was no significant difference between extract-treated cells alone, compared with the normal group, except that at 250 µg/ml *S. fruticosa*, IL-1β was below the detection limit of the kit (64 pg/ml).

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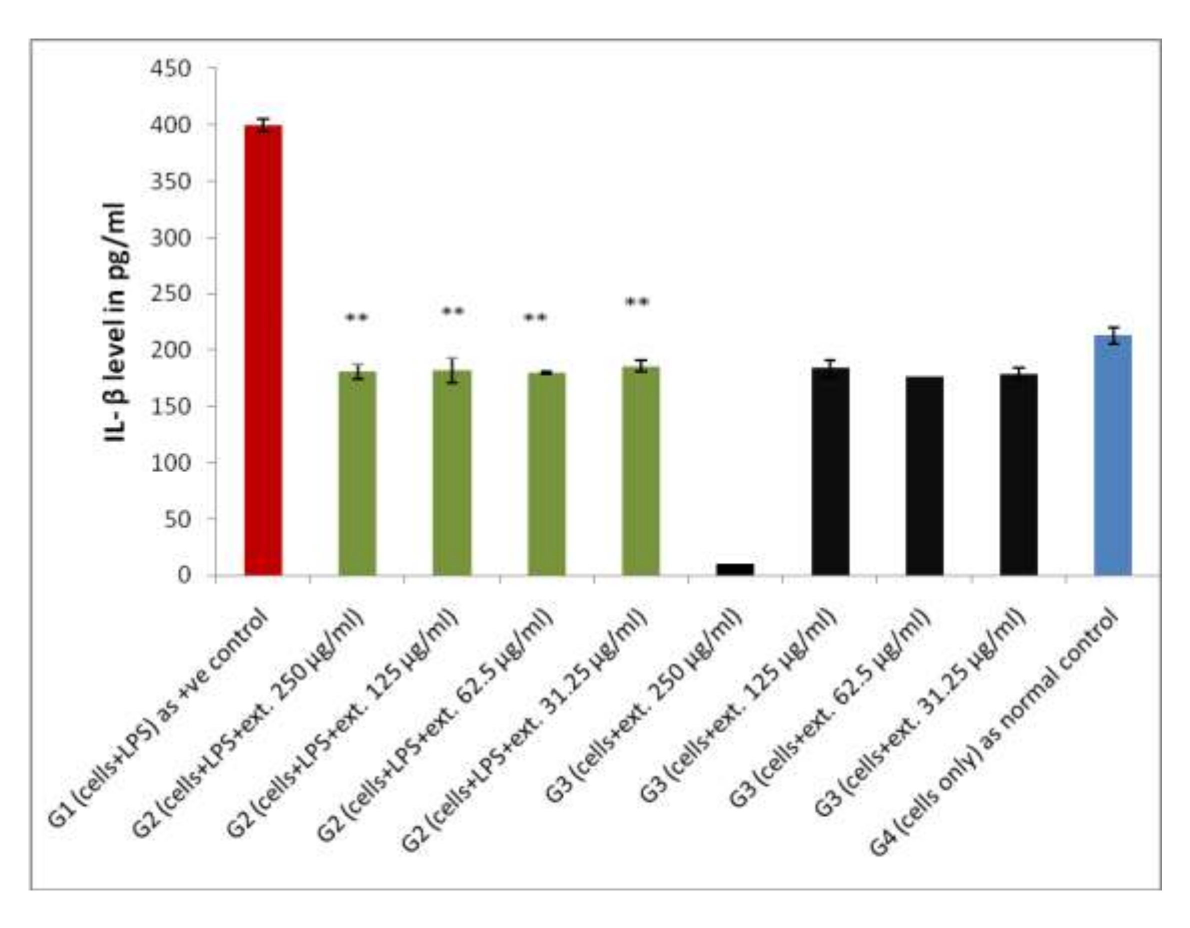


Figure 3. Effect of *S. fruticosa* on IL-1β production by RAW 264.7 cells G1: Positive control (red column); G2: Treated group with *S .fruticosa* and LPS (green columns); G3: Extract-treated group without LPS (black columns); G4: Normal group (blue column) ** $p < 0.01$ compared to the normal to the positive control group.

4.1.2. Effect of *S. fruticosa* on RAW 264.7 cells viability

To study the effect of *S. fruticosa* on the viability of RAW 264.7 cells, cells were incubated with different concentrations of the plant extract (31.25-250 µg/ml) as the test group and compared with the normal control group of cells that were incubated only with RPMI-1640 medium in 5% CO_2 incubator with a humidified atmosphere at 37 °C for 18 hours same as with cytokine production experiment. Cell viability was not affected at all extract concentrations used, almost they were equal as determined by MTT assay, except for 250 µg/ml concentration, they have higher O.D reading (Fig.4 A). When the incubation period extended to 72 hours of another plate, *S. fruticosa* exhibited significant anti-proliferative effect on RAW 264.7 cells treated with extract

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concentrations between 62.5-250 µg/ml in a dose-dependent manner compared to the normal untreated group ($p < 0.01$) and the lowest concentration 31.25 µg/ml has no significant effect on proliferation of RAW 264.7 cells as determined by MTT assay (Fig.4 B).

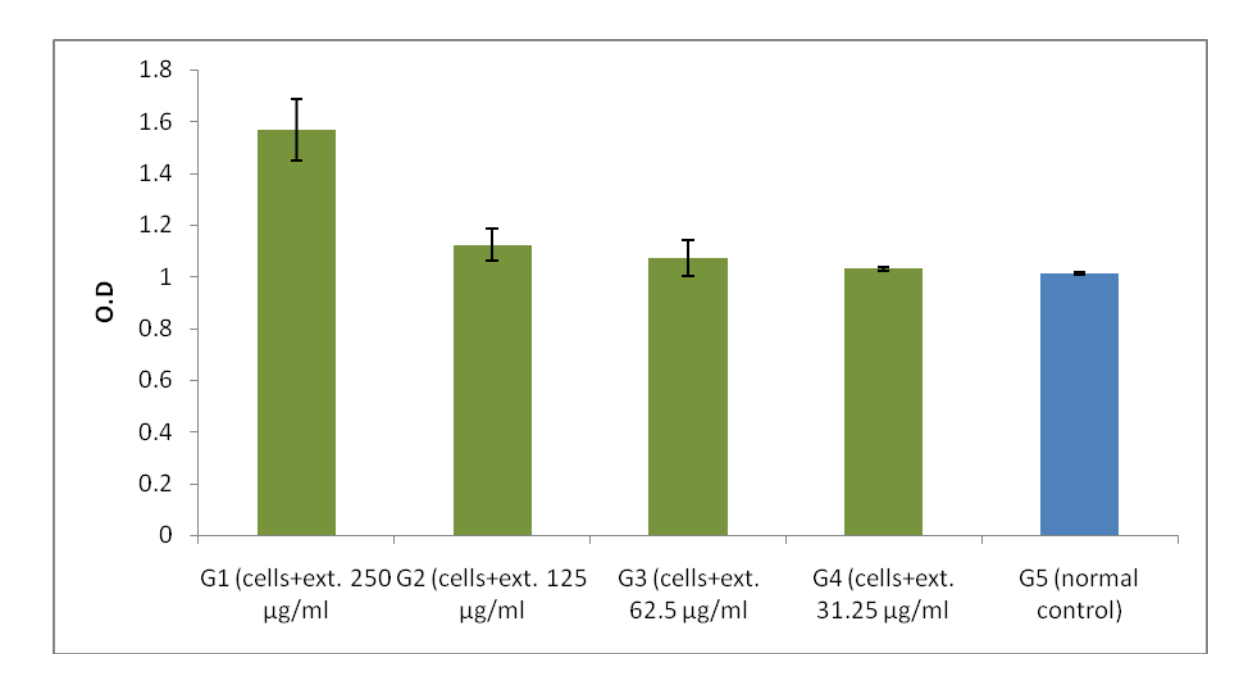


Figure 4 A. Effect of *S. fruticosa* on viability of RAW 264.7 cells G1, G2, G3 and G4 (green columns) are cell groups treated with plant extract at various concentrations (31.25-250 µg/ml) for 18 hours, G5 (blue column) is the normal untreated group.

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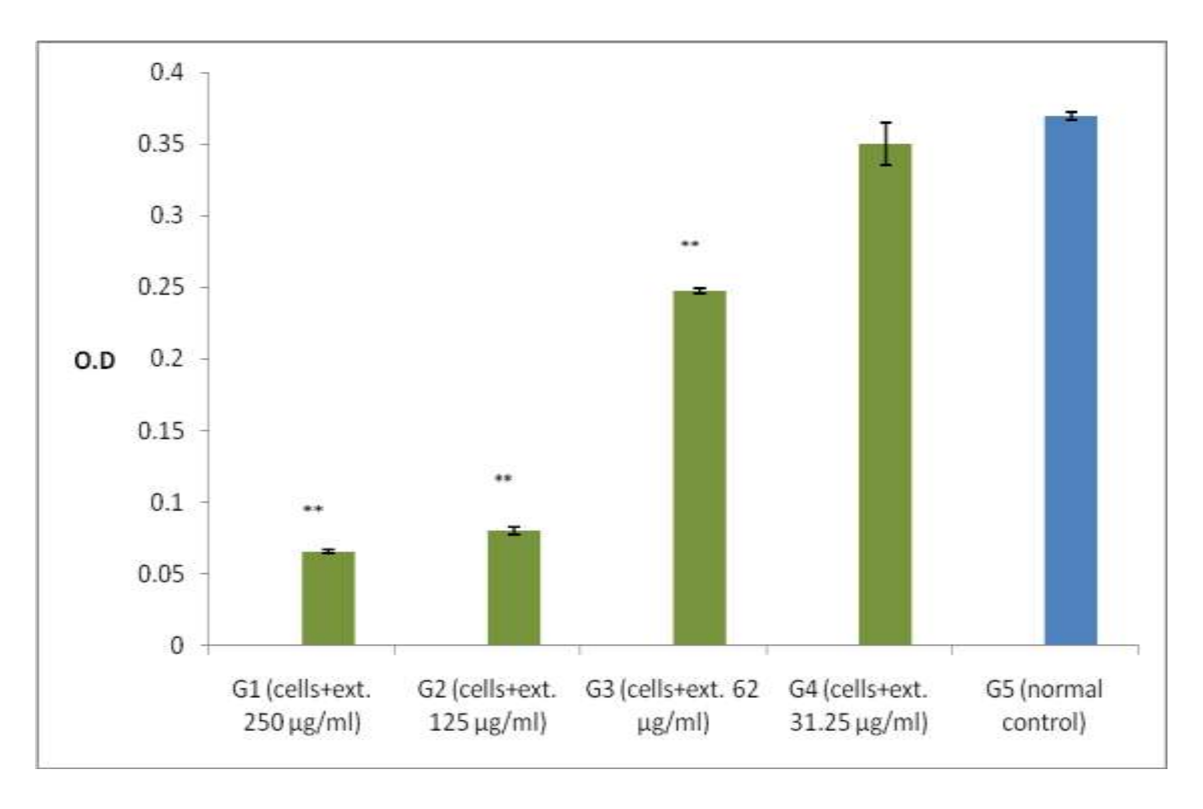


Figure 4 B. Effect of *S. fruticosa* on proliferation of RAW 264.7 cells G1, G2, G3 and G4 (green columns) are cell groups treated with plant extract at various concentrations (31.25-250 µg/ml) for 72 hours, G5 (blue column) is the normal untreated group ** $p < 0.01$ compared to the normal control group.

4.1.3. Effect of *S. fruticosa* on TNF-α, IL-1β and IL-6 production by peritoneal macrophages

As shown in Fig. 5, treatment with *S. fruticosa* strongly inhibited the production of TNF-α by peritoneal macrophages at all concentrations used (250, 125, 62.5 and 31.25 µg/ml). The reduction was 96%, 92%, 65% and 62% respectively compared to the single-LPS stimulated cells (positive control), TNF-α increased by 37-fold in the presence of LPS only, reaching 1758 pg/ml, then falling to only 57 pg/ml at the highest concentration of the plant extract (250 µg/ml) which was very close to the normal unstimulated cells and to 133, 610 and 653 pg/ml at the other concentrations respectively ($p < 0.01$).

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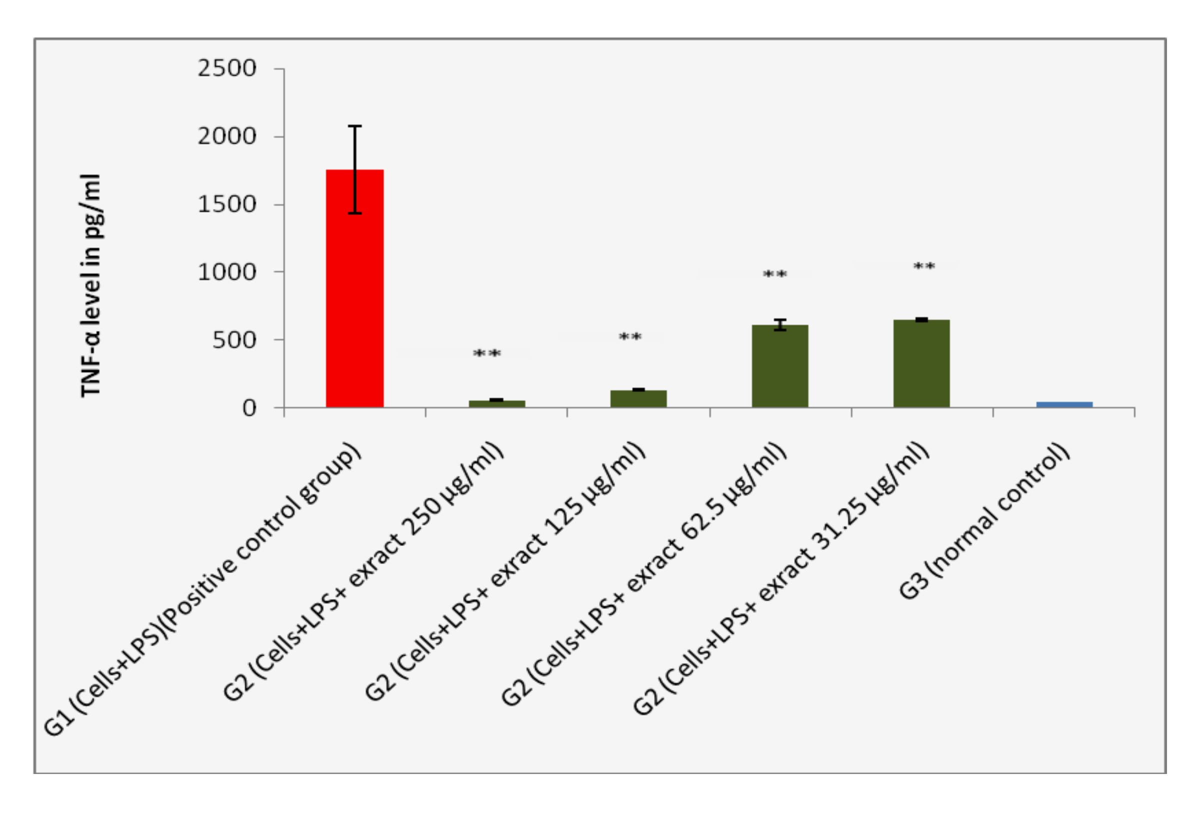


Figure 5. Effect of *S. fruticosa* on TNF-α production by peritoneal macrophages G1: Treated groups with *S. fruticosa* and LPS (green columns); G1: Positive control group LPS only (red column); G3: Normal group ** $p < 0.01$ compared to the positive control group.

Surprisingly, IL-6 production increased by 190-fold in the single-LPS stimulated cells (positive control group) reaching 45454 pg/ml as shown in Fig. 6, then falling to only 229 and 325 pg/ml at *S. fruticosa* extract concentrations 250 and 125 µg/ml respectively. The IL-6 also tremendously decreased to 4048 and 8139 pg/ml at concentrations 62.5 and 31.25 µg/ml respectively. The inhibition ratios reached up to 99.5%, 99.2%, 91% and 82% respectively ($p < 0.01$).

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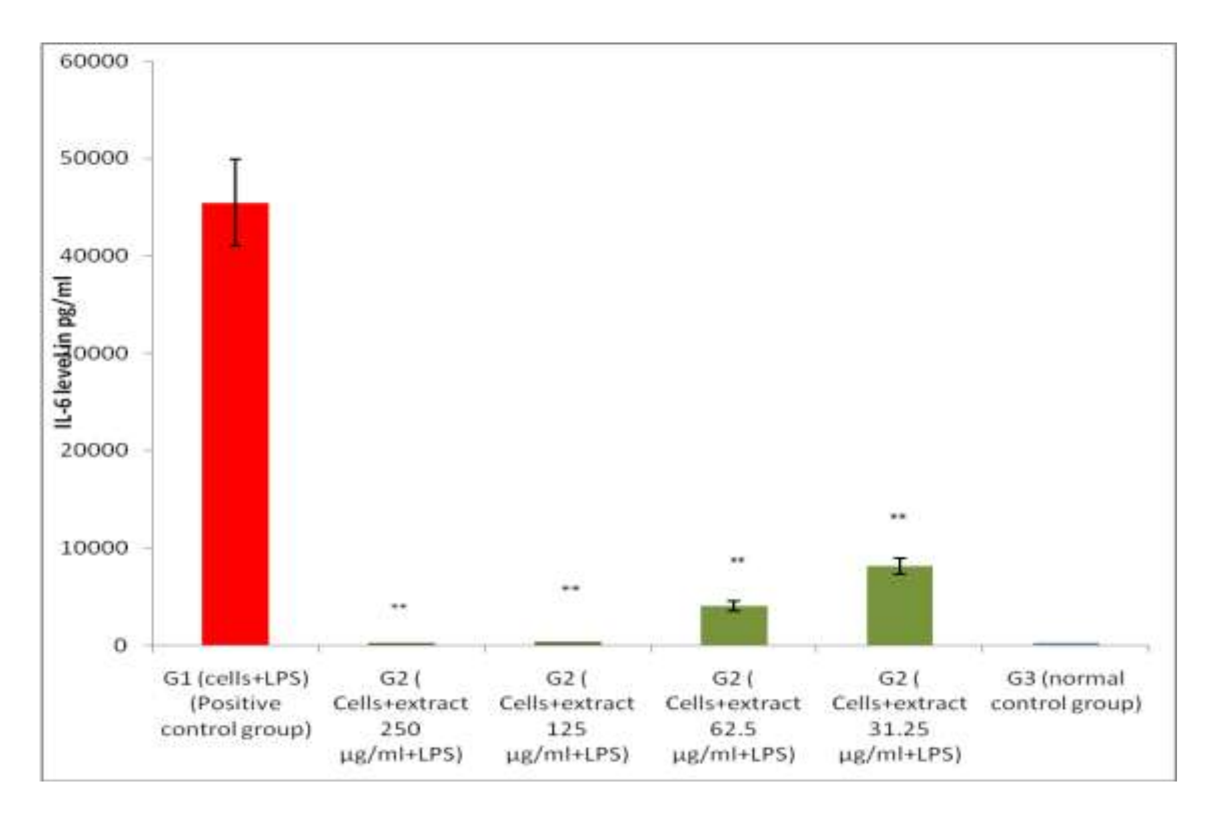


Figure 6. Effect of *S. fruticosa* on IL-6 production by peritoneal macrophages G1: Treated groups with *S. fruticosa* and LPS (green columns); G1: Positive control group LPS only (red column); G3: Normal group ** p <0.01 compared to the positive control group.

The production of IL-1β by single-LPS stimulated peritoneal macrophages was slightly increased, reaching 309 pg/ml, then falling to 153, 149 and 184 pg/ml in *S. fruticosa* treated cells at concentrations 250, 125 and 62.5 pg/ml with inhibition ratios up to 50%, 51% and 40% respectively ($p < 0.05$). At the lowest concentration of the plant extract (31.25 µg/ml), IL-1β level was 194 pg/ml which was statistically not significant (Fig. 7).

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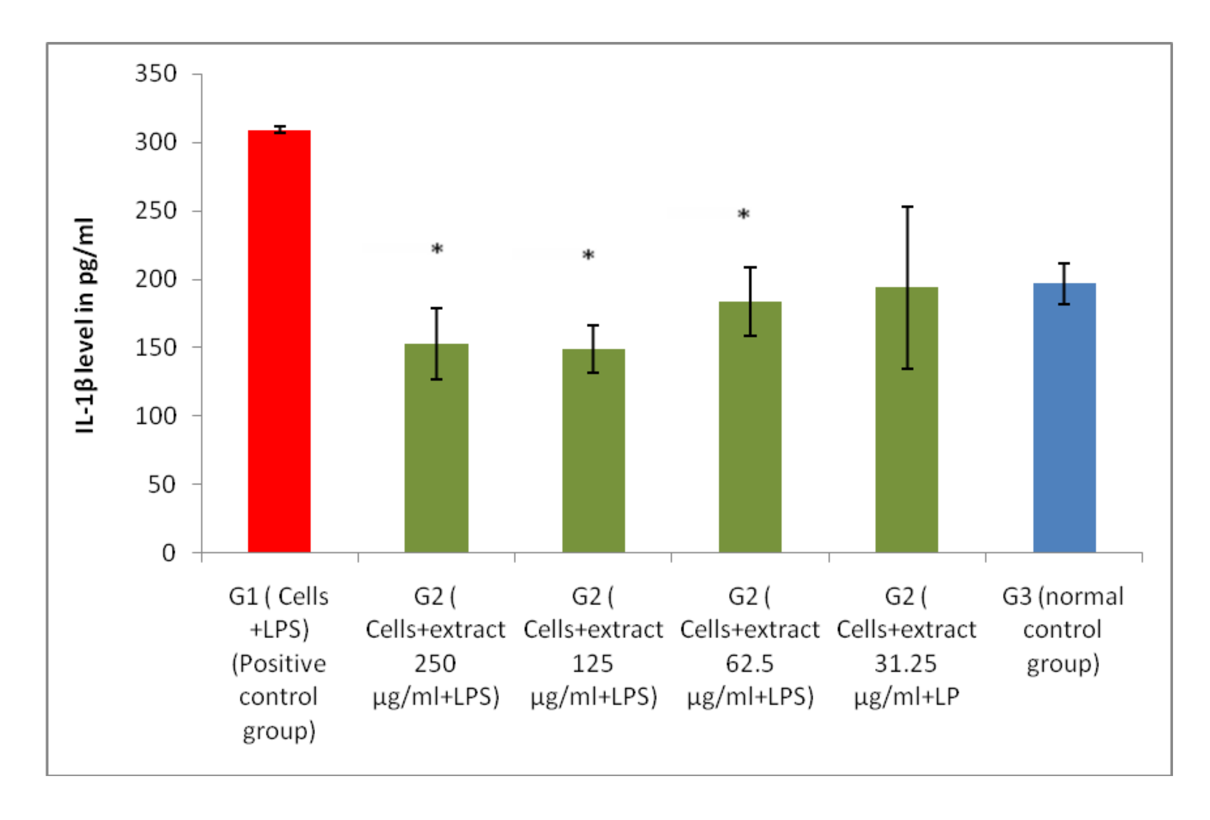


Figure 7. Effect of *S. fruticosa* on IL-1β production by peritoneal macrophages G1: Treated groups with *S. fruticosa* and LPS (green columns); G1: Positive control group LPS only (red column); G3: Normal group * $p < 0.05$ compared to the positive control group.

4.1.4. Effect of *S. fruticosa* on peritoneal macrophages viability

Viabilty of peritoneal macrophages was not affected at all *S. fruticosa* extract concentrations (31.25-250 µg/ml) compared to the normal untreated cells after 18 hours incubation ($p > 0.2$), as shown in Fig. 8.A.

While, when the incubation period extended for 72 hours, only at the highest concentration (250 µg/ml), *S. fruticosa* exhibited anti-proliferative effect ($p < 0.05$) on peritoneal macrophages as shown in Fig. 8.B.

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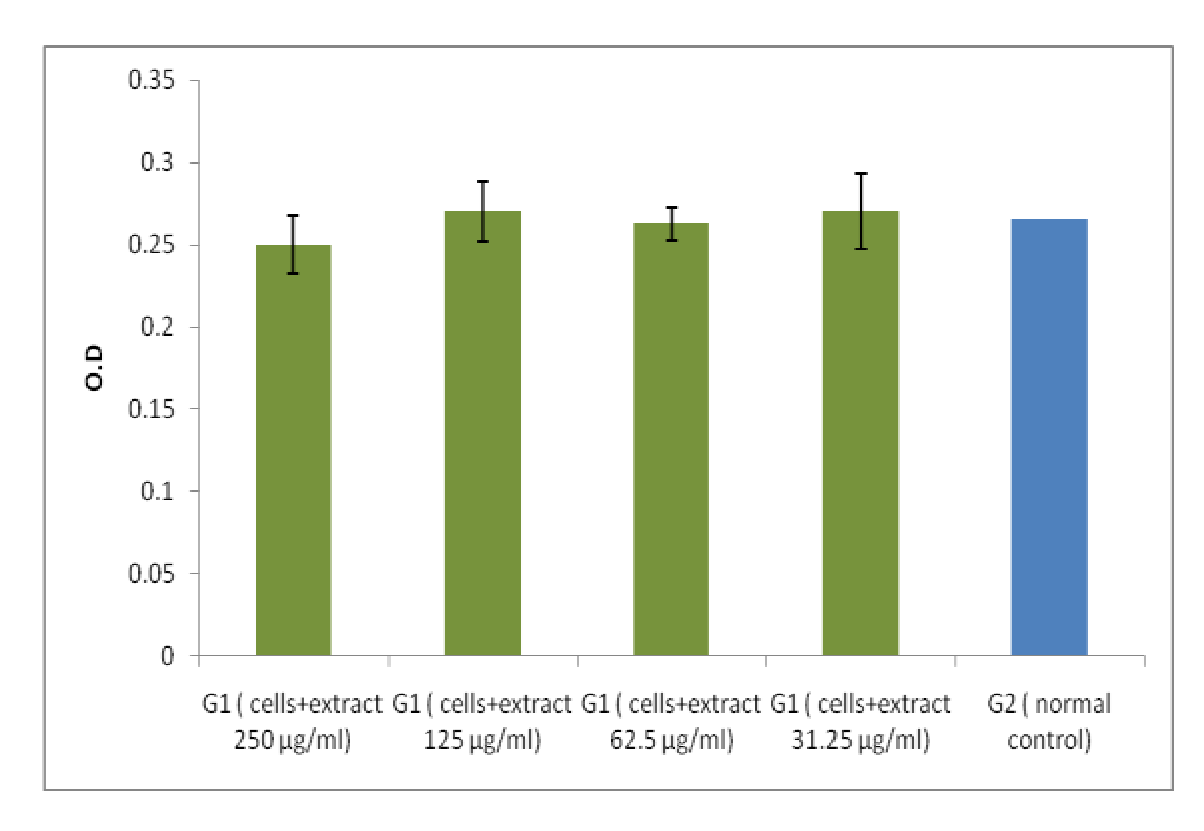


Figure 8. A. Effect of *S. fruticosa* on viability of peritoneal macrophages as determined by MTT assay after 18 hours incubation.

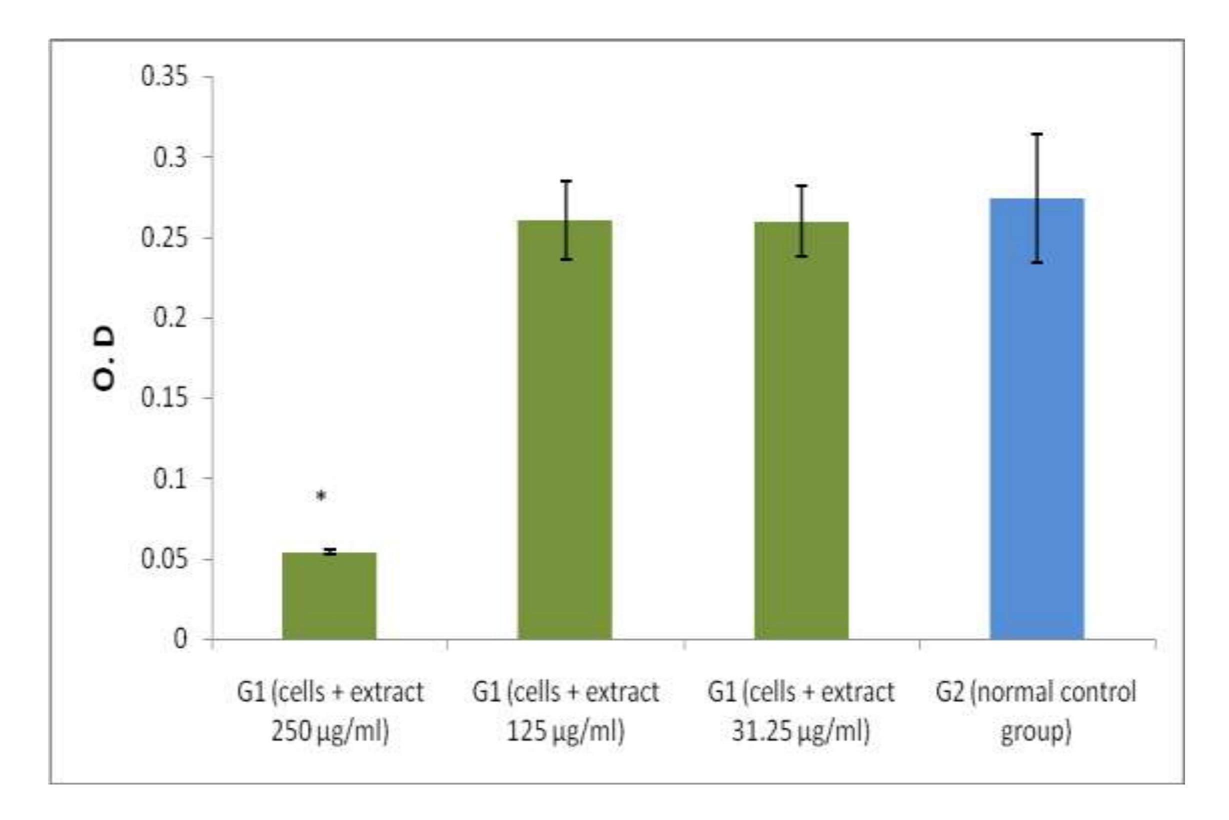


Figure 8. B. Effect of *S. fruticosa* on proliferation of peritoneal macrophages as determined by MTT assay after 72 hours incubation * $p < 0.05$ compared to the normal control group.

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4.1.5. Effect of *S. fruticosa* on TNF-α, IL-1β and IL-6 production by BMDM

The production of TNF-α by BMDM was suppressed up to 71% at the highest concentration of *S. fruticosa* (250 µg/ml) only compared to the single-LPS stimulated cells (the positive control). TNF- α level was 4653 pg/ml in LPS-stimulated cells with a 6-fold increase compared to normal cells, and then falling to 1337 pg/ml ($p < 0.05$) at 250 µg/ml concentration of the plant extract as shown in Fig.9. At concentration 125 µg/ml the decrease in TNF-α was only 25% (3473 pg/ml) which was statistically not significant as well as for the lowest concentration (31.25 µg/ml).

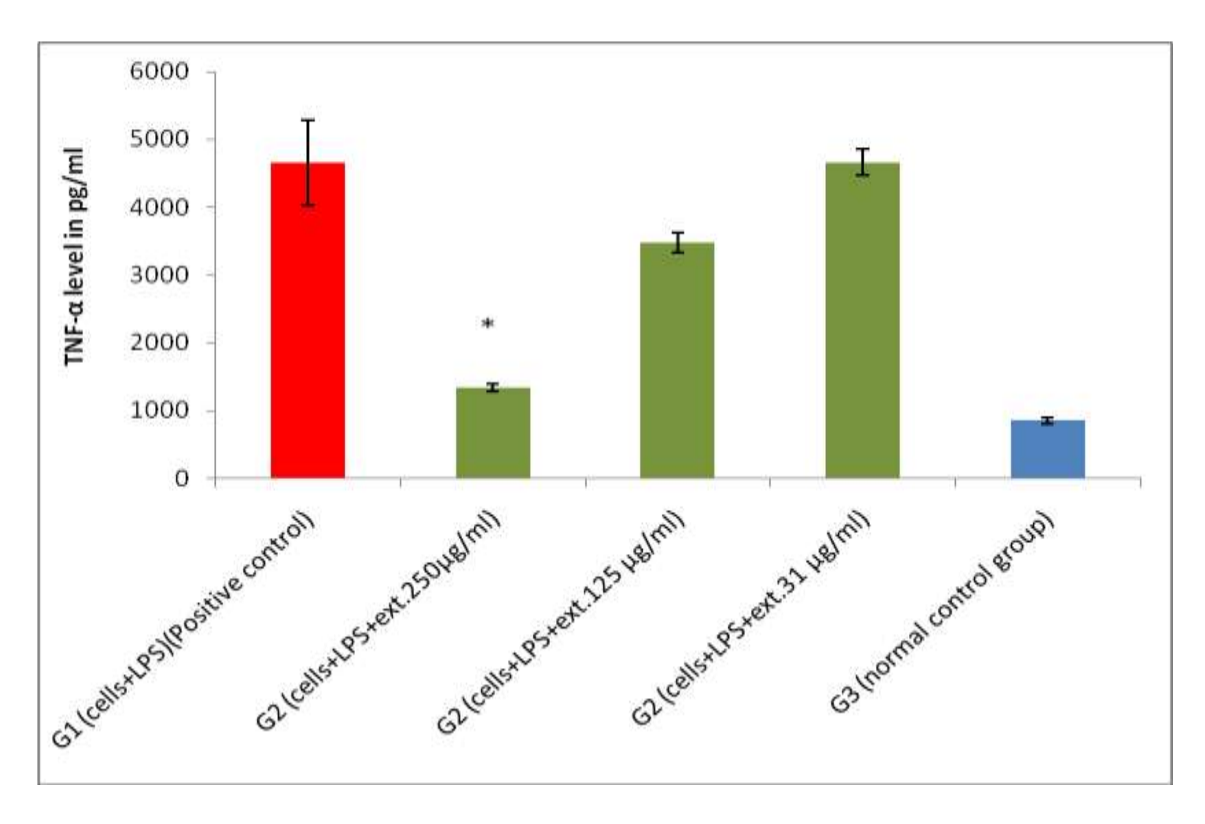


Figure 9. Effect of *S. fruticosa* on TNF-α production by BMDM G1: Treated groups with *S. fruticosa* and LPS (green columns); G1: Positive control group LPS only (red column); G3: Normal group * $p < 0.05$ compared to the positive control group.

Extremely surprising, IL-6 production by BMDM was completely inhibited by *S. fruticosa* at concentrations 250 and 125 µg/ml of the plant extract, IL-6 was below the

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detection limit of the kit (64 pg/ml), while, reached 12195 pg/ml in single-LPS stimulated cells (the positive control) and it was only 251 pg/ml in the normal cells. Also, at the 31.25 µg/ml of the plant extract, IL-6 was inhibited up to 68% (3850 pg/ml) and ($p < 0.01$) as shown in Fig.10.

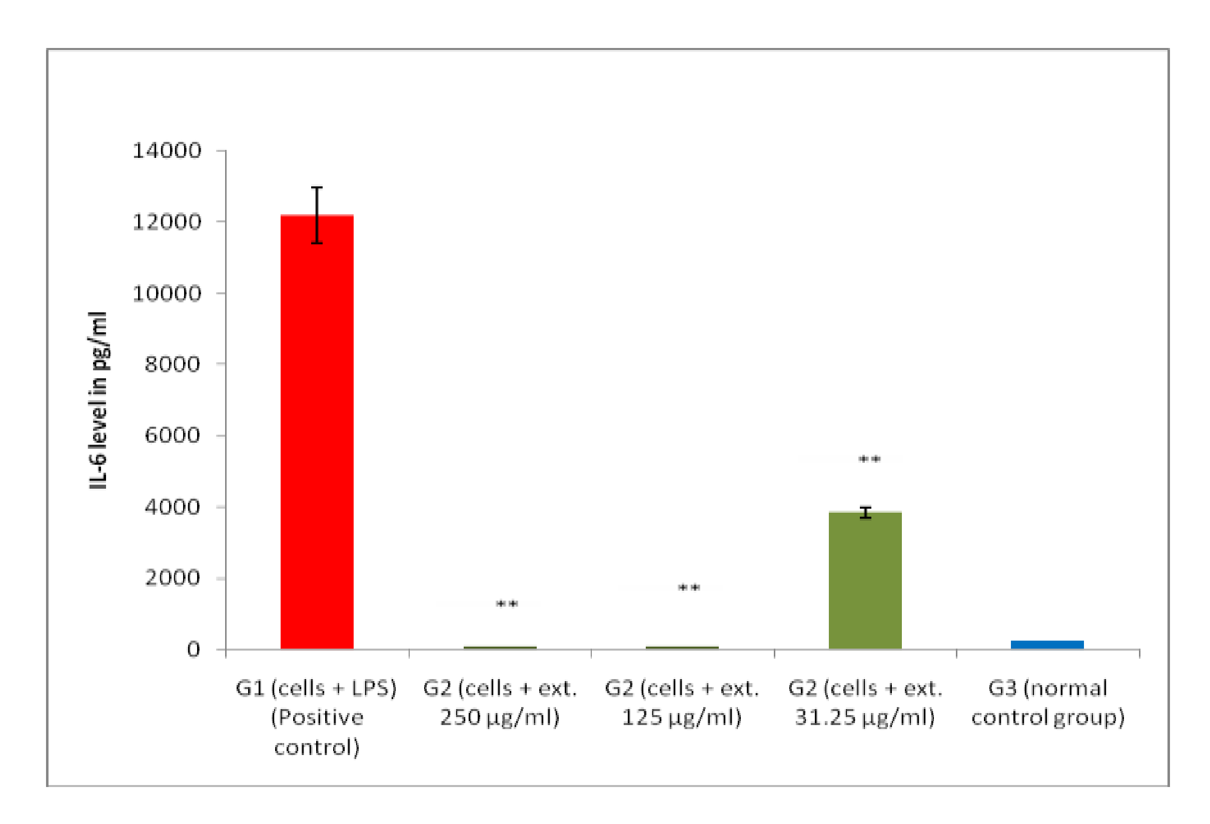


Figure 10. Effect of *S. fruticosa* on IL-6 production by BMDM G1: Treated groups with *S. fruticosa* and LPS (green columns); G1: Positive control group LPS only (red column); G3: Normal group ** p < 0.01 compared to the positive control group.

In Fig.11, IL-1β secretion by BMDM was below the detection limit of the kit (64 pg/ml) at all concentrations 250, 125 and 31.25 µg/ml of *S. fruticosa* extract. And it was only 334 pg/ml in the single-LPS stimulated cells (the positive control) with 3.5-fold increase compared to the normal cells (94 pg/ml) and ($p < 0.05$).

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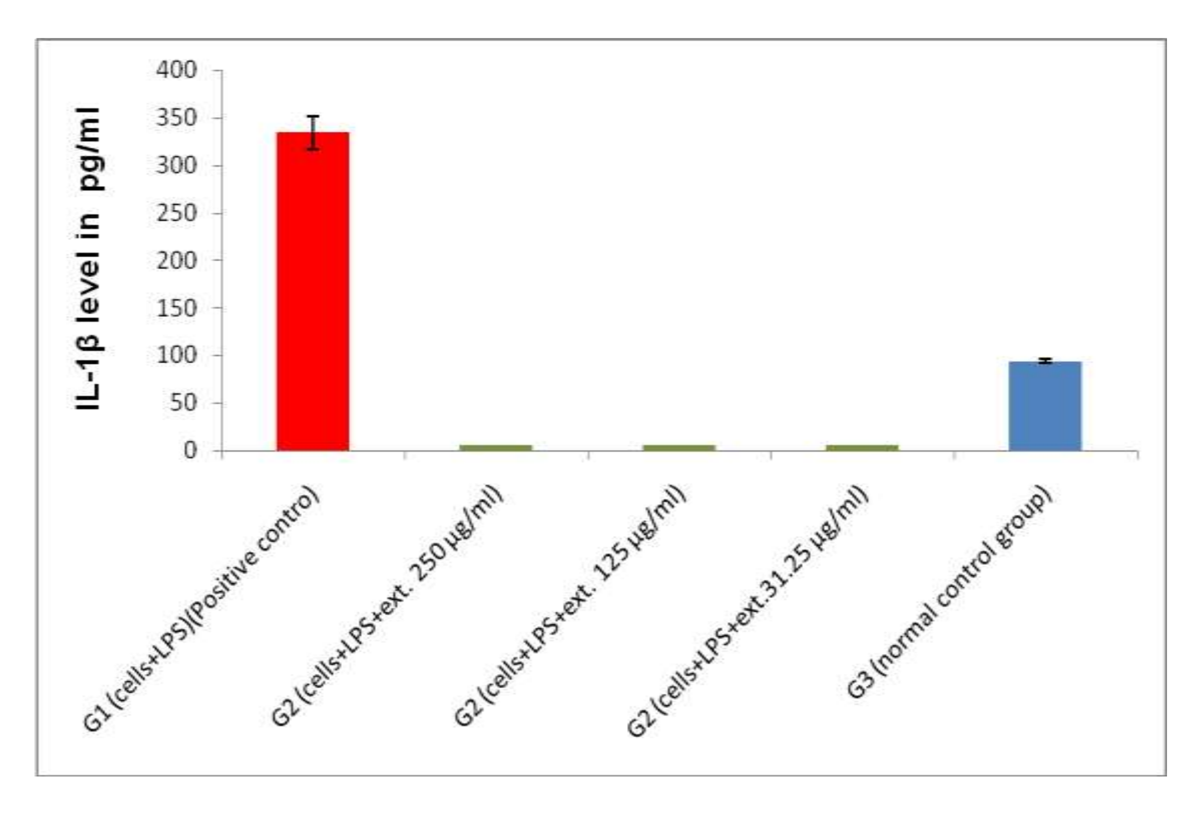


Figure 11. Effect of *S. fruticosa* on IL-1β production by BMDM G1: Treated groups with *S. fruticosa* and LPS (disappeared because their values were below the detection limit); G1: Positive control group LPS only (red column); G3: Normal group.

4.1.6. Effect of *S. fruticosa* on viability of BMDM

Viabilty of BMDM was not affected at all extract concentrations of *S. fruticosa* (250, 125 and 31.25 µg/ml) compared to the normal cells after 18 hours incubation. Also, viability was not affected by LPS (1 µg/ml) stimulation under same conditions ($p > 0.1$) as shown in Fig.12.

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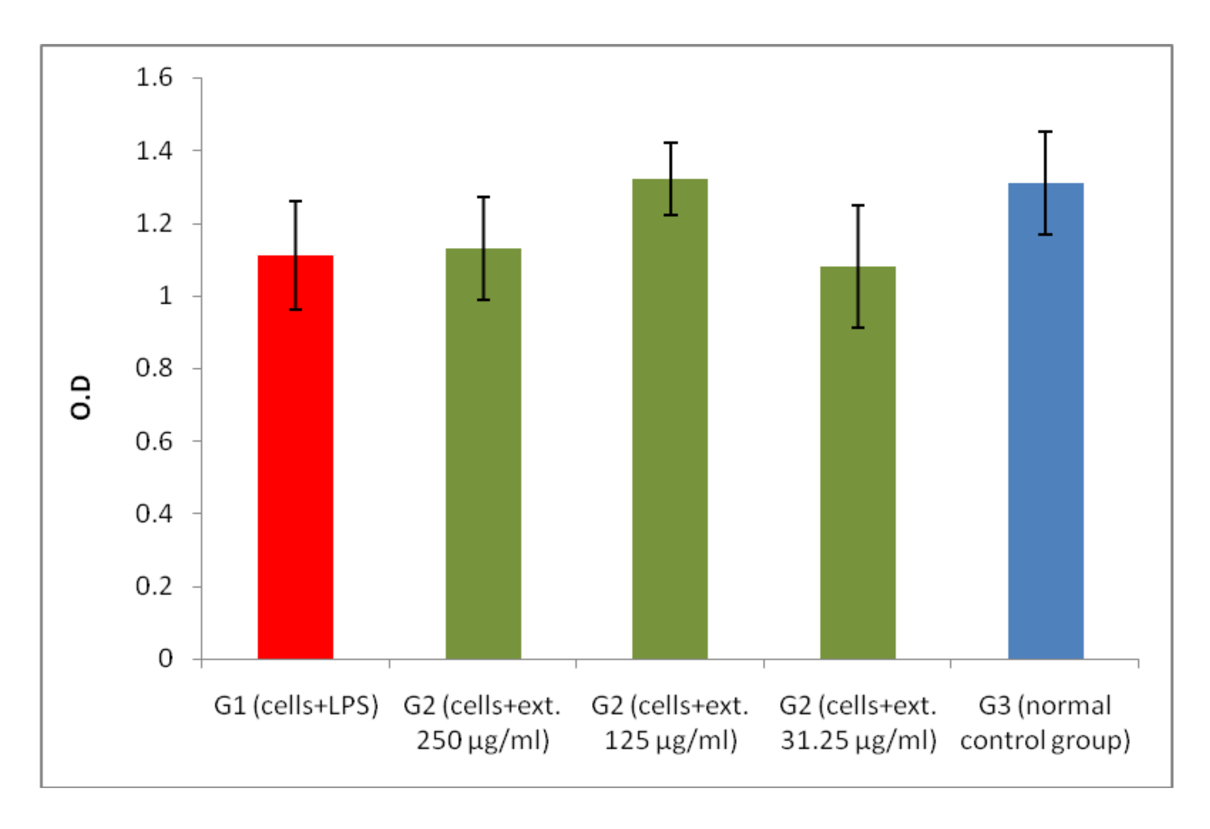


Figure 12. Effect of *S. fruticosa* on viability of BMDM as determined by MTT assay after 18 hours incubation.

4.1.7. Effect of *S. fruticosa* on TNF-α, IL-1β and IL-6 production by splenocytes

The production of TNF-α by single-ConA stimulated splenocytes (the positive control) reached a level of 313 pg/ml then falling to 189 pg/ml at the highest concentration of *S. fruticosa* extract used (250 µg/ml) with inhibition ratio of 39% . The other concentrations used exhibited an inhibition ratios between 17-22 % with a p value < 0.05 for all concentrations.

The plant extract has no effect on the TNF-α baseline level produced by the normal cells (Fig.13).

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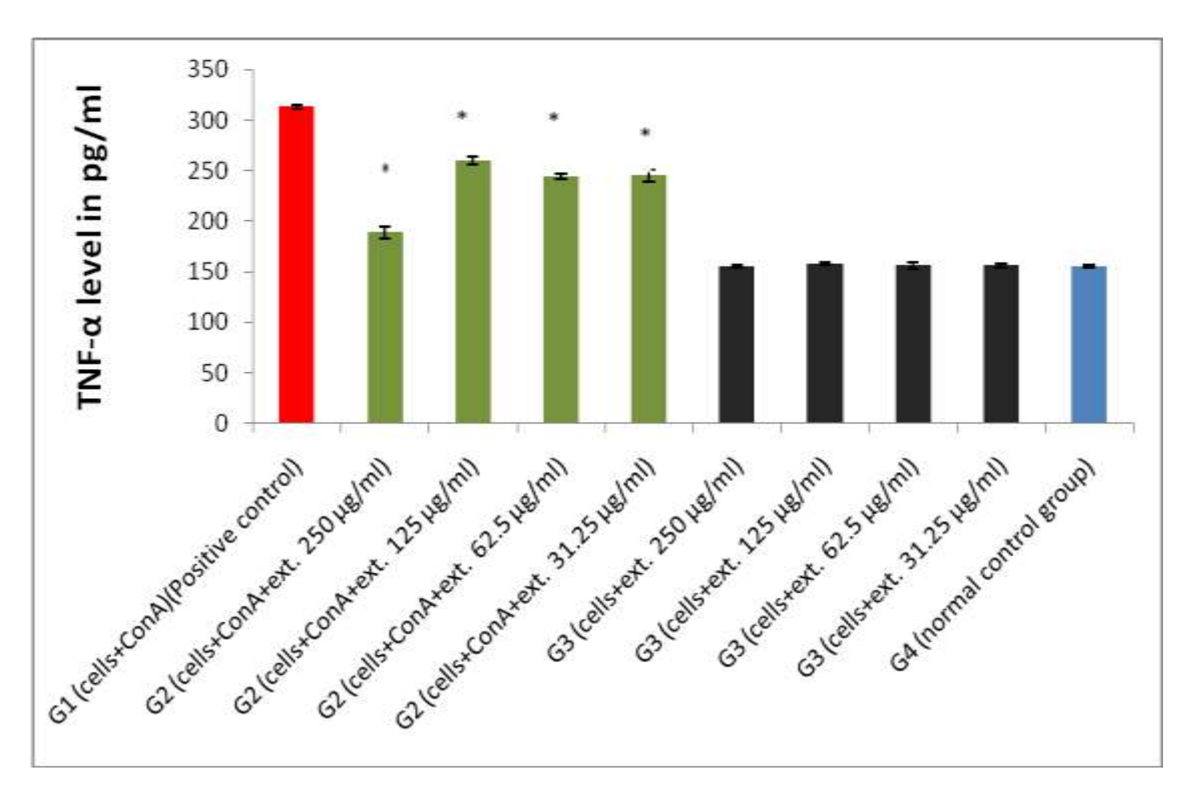


Figure 13. Effect of *S. fruticosa* on TNF-α production by splenocytes G1: Positive control (red column); G2: Treated group with *S .fruticosa* and ConA (green columns); G3: Extract-treated group without ConA (black columns); G4: Normal group (blue column) * $p < 0.05$ compared to the positive control group.

The inhibitory effect of *S. fruticosa* on IL-6 production by splenocytes was tremendous, it was decreased up to 62% compared to the single-ConA stimulated cells (the positive control) reaching 2968 pg/ml, then falling to 1129 pg/ml at the highest concentration of the plant extract used (250 µg/ml) and at concentrations 125 and 62.5 µg/ml IL-6 decreased up to 38% and 26% respectively with a p value < 0.01, but at the lowest concentration used (31.25 µg/ml) there was no significant decrease in IL-6 level. Also, *S. fruticosa* treatment alone did not affect IL-6 level compared to the normal cells (Fig.14).

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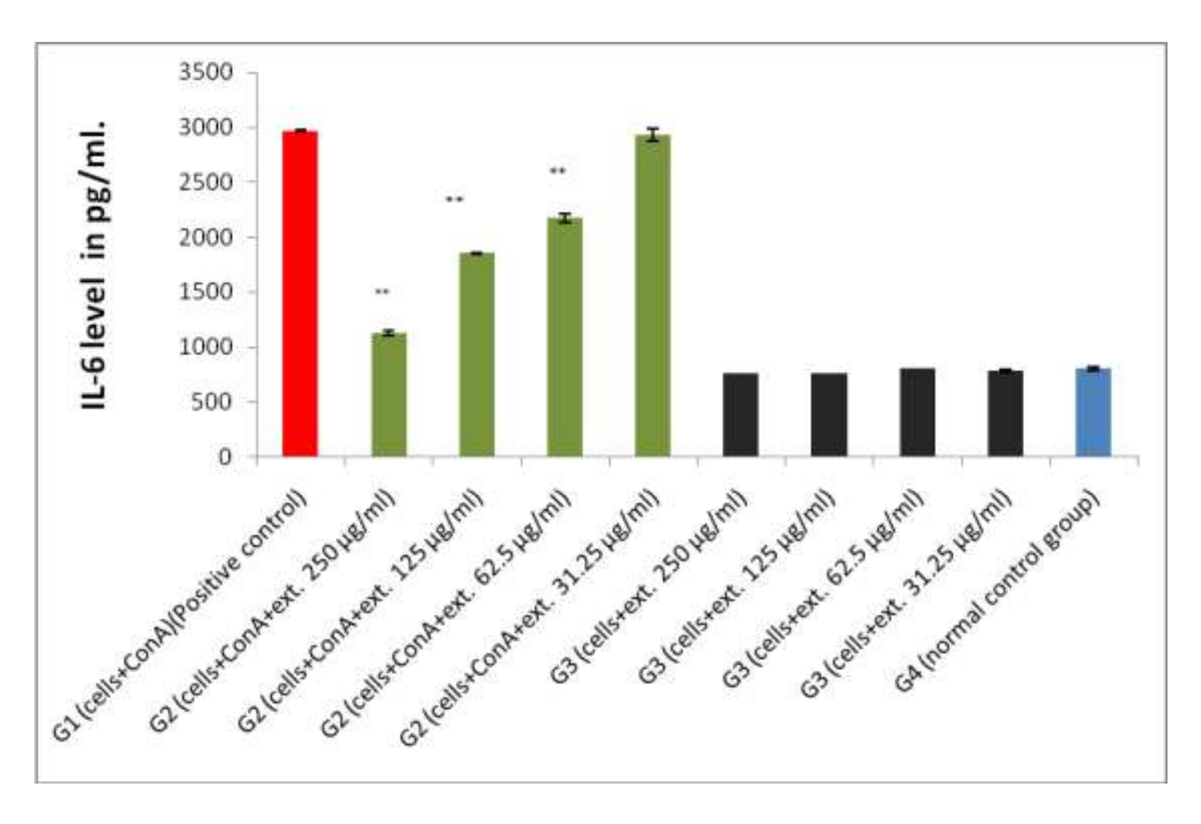


Figure 14. Effect of *S. fruticosa* on IL-6 production by splenocytes G1: Positive control (red column); G2: Treated group with *S .fruticosa* and ConA (green columns); G3: Extract-treated group without ConA (black columns); G4: Normal group (blue column) ** $p < 0.01$ compared to the positive control group.

As shown in Fig.15, IL-1β was decreased in splenocytes stimulated with ConA and treated with different concentrations of *S. fruticosa* extract. All concentrations used inhibited IL-1β production by approximately 25% compared to the positive control group ($p < 0.05$). No significant effect of the plant extract treatment alone on IL-1β in normal cells.

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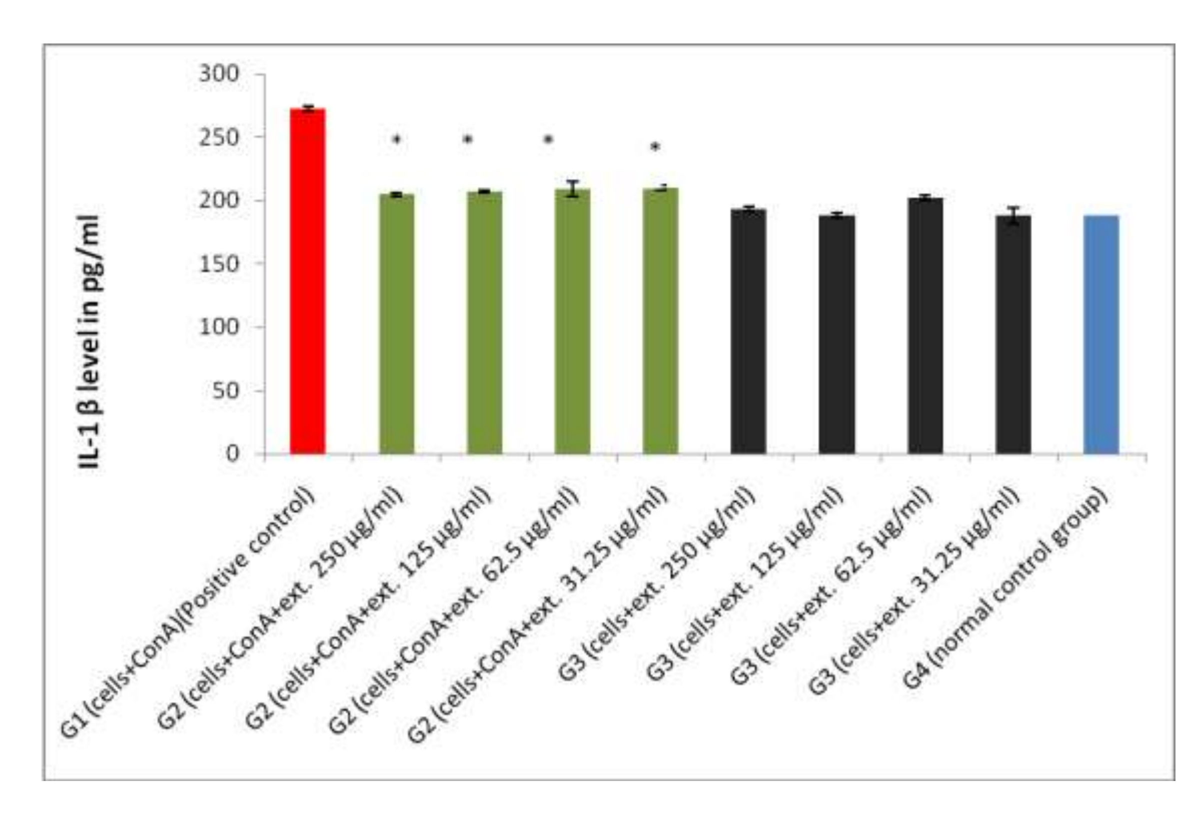


Figure 15. Effect of *S. fruticosa* on IL-1β production by splenocytes G1: Positive control (red column); G2: Treated group with *S .fruticosa* and ConA (green columns); G3: Extract-treated group without ConA (black columns); G4: Normal group (blue column) * $p < 0.05$ compared to the positive control group.

4.1.8. Effect of *S. fruticosa* on viability of splenocytes

Viabilty of splenocytes was not affected by *S. fruticosa* treatment at all concentrations used whether with ConA or alone after incubation for 18 hours as well as not affected by ConA alone as shown in Fig.16A. However, when the incubation period extended to 72 hours, *S. fruticosa* exhibited significant inhibitory effect on splenocytes proliferation at concentrations 250 and 125 µg/ml with ConA ($p < 0.01$) and at concentrations 62.5 and 31.25 µg/ml with ConA ($p < 0.05$) compared to the single ConA-stimulated group. Also, *S. fruticosa* had a strong anti proliferative effect on splenocytes treated with extract at concentrations (62.5-250 µg/ml) with a p value < 0.01 and no such effect at the lowest conentration used (31.25 µg/ml) compared to the normal untreated cells as shown in Fig.16B.

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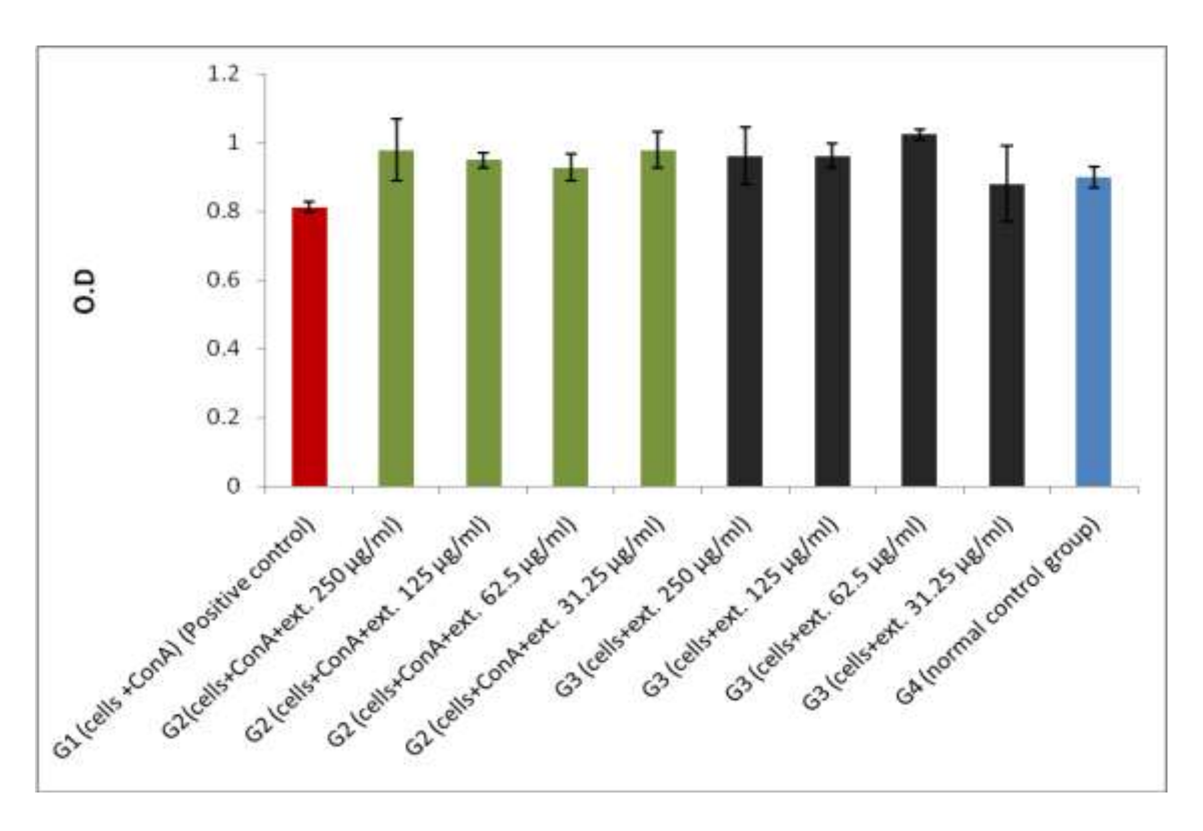


Figure 16A. Effect of *S. fruticosa* on viability of splenocyte as determined by MTT assay after 18 hours incubation.

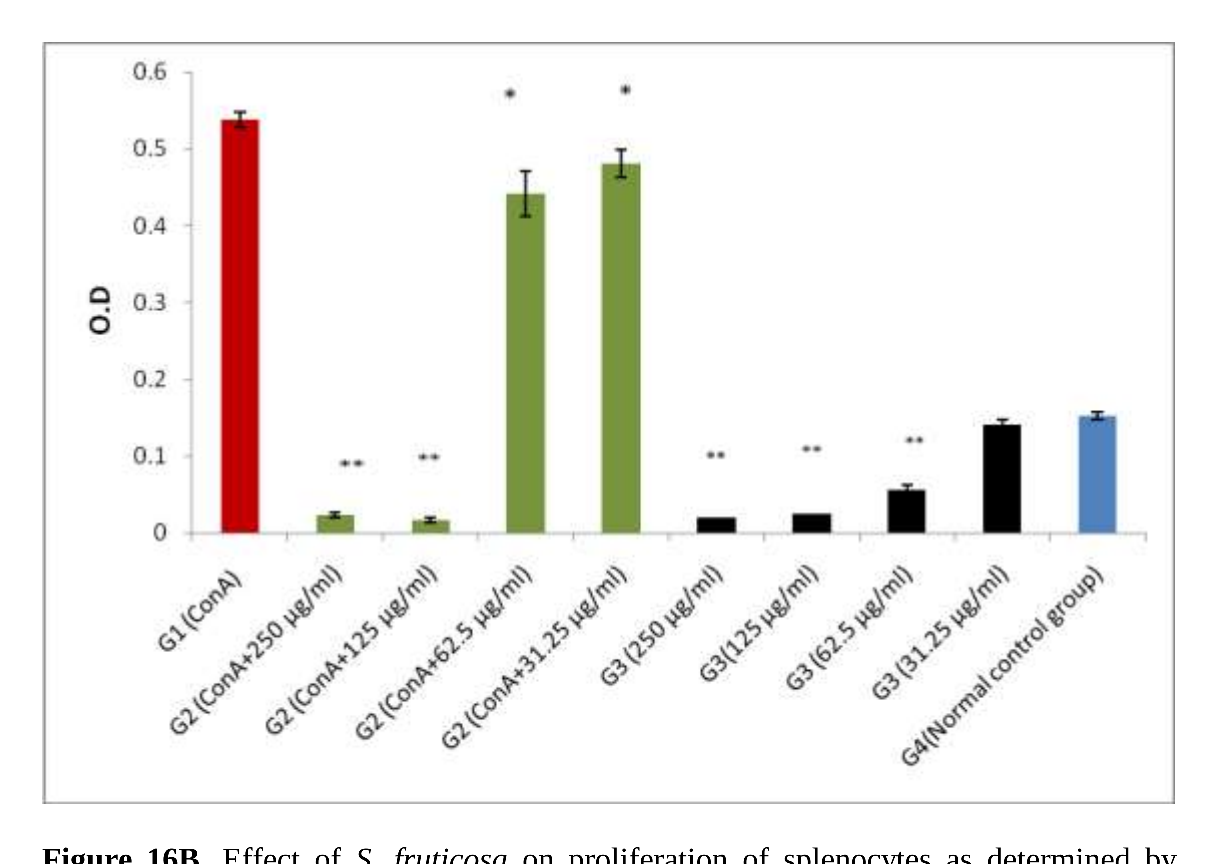


Figure 16B. Effect of *S. fruticosa* on proliferation of splenocytes as determined by MTT assay after 72 hours incubation ** $p < 0.01$, * $p < 0.05$.

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4.2. Effect of *S. fruticosa* on serum TNF-α, IL-6 and IL-1β release in LPS-challenged mice

This experiment was conducted to investigate changes of TNF-α, IL-6 and IL-1β secretion in LPS-challenged Balb/c mice after 2 hours of i.p injection of 25 µg LPS/mouse (1 µg LPS/gram body weight) and the effect of pretreatment with *S. fruticosa* in mice before LPS stimulation on such changes. The results on TNF-α production are illustrated in Fig.17. The plant extract highly supressed TNF-α release in the serum of mice group that was treated with the extract before one hour of LPS injection, the reduction level reached up to 85% compared to the LPS-stimulated group (the positive control) reaching 5980 pg/ml, then falling to only 913 pg/ml ($p < 0.01$).

The baseline level was not affected in the single extract-treated mice compared to the normal control group that was only injected with the vehicle PBS.

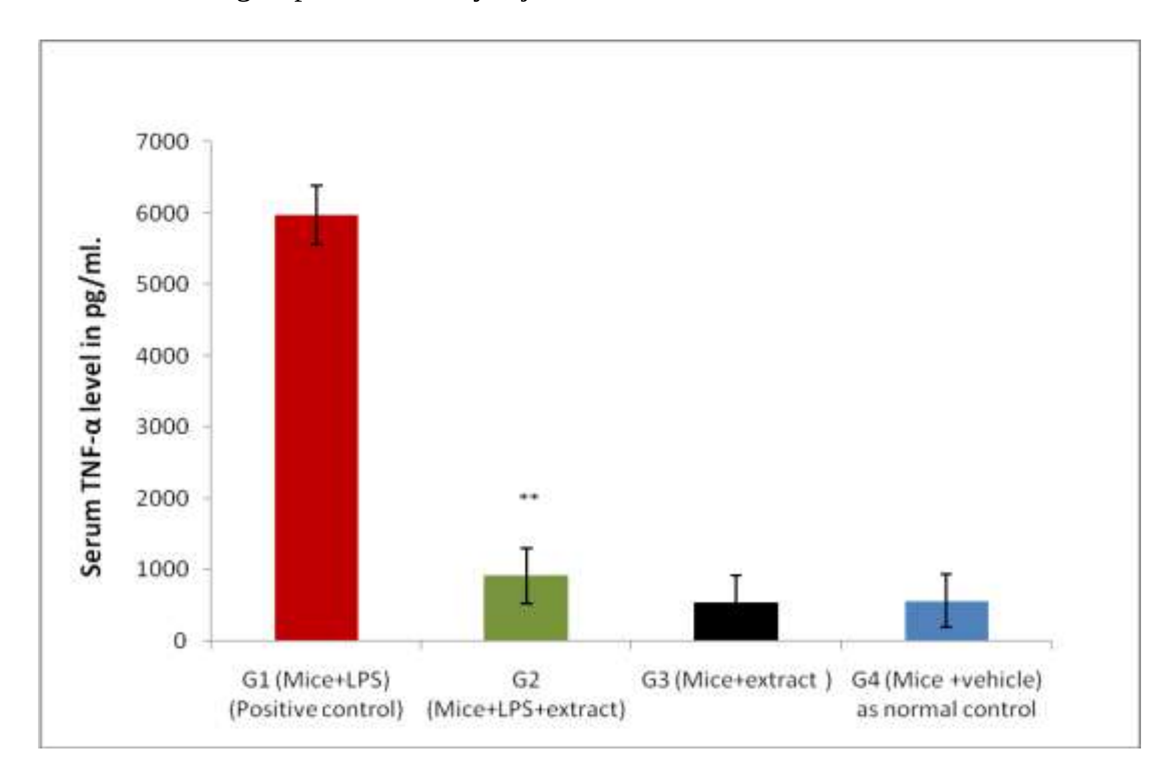


Figure 17. Effect of *S. fruticosa* on serum TNF-α of Balb/c mice. G1: mice only stimulated with LPS(red column); G2: mice treated with *S. fruticosa* extract and stimulated with LPS (green column); G3: mice only treated with extract (black column); G4: mice only injected with the vehicle PBS (blue column) ** $p < 0.01$ compared to the positive control group.

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The serum level of IL-6 was decreased up to 56% in *S. fruticosa* treated mice compared to the LPS-stimulated group, reaching 55000 pg/ml, and then falling to 24000 pg/ml ($p < 0.01$). There was no significant difference in the baseline level between single extract-treated mice and the normal control group as shown in Fig.18.

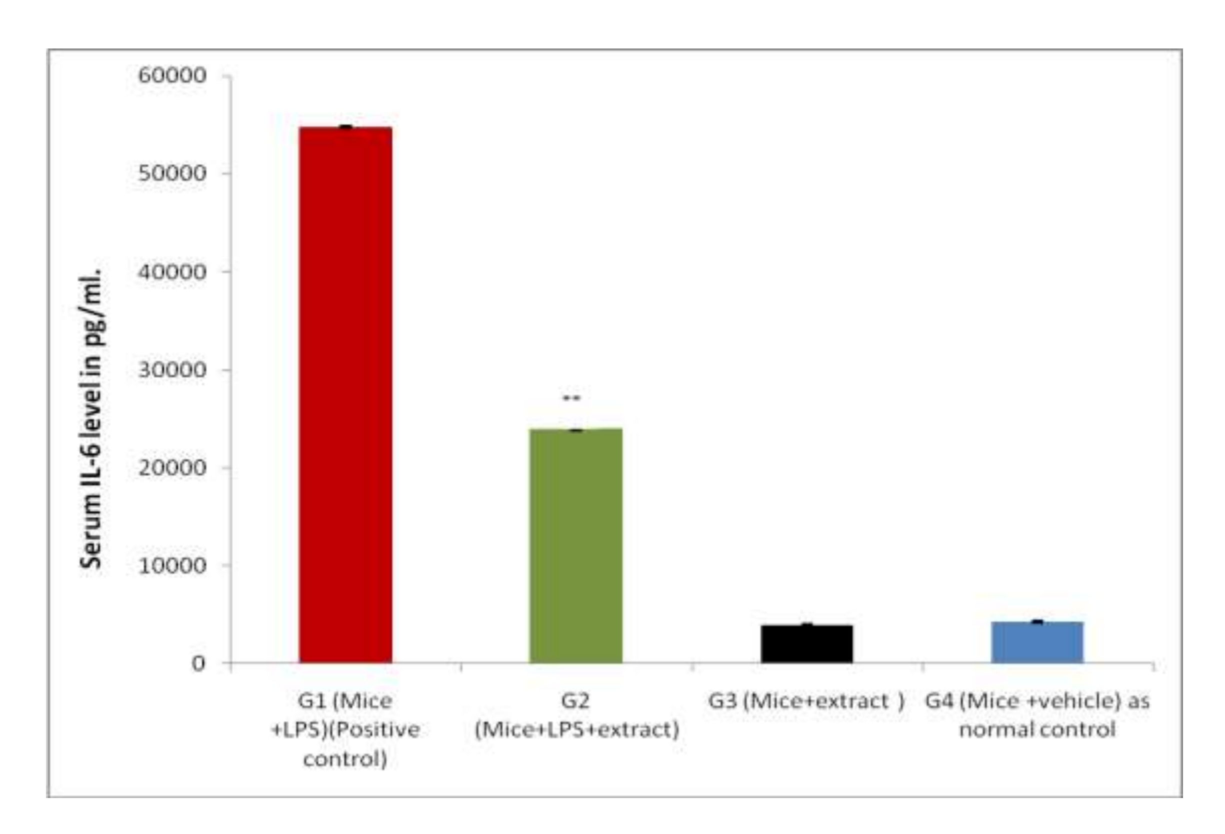


Figure 18. Effect of *S. fruticosa* on serum IL-6 of Balb/c mice. G1: mice only stimulated with LPS(red column); G2: mice treated with *S. fruticosa* extract and stimulated with LPS (green column); G3: mice only treated with *S. fruticosa* extract (black column); G4: mice only injected with the vehicle PBS (blue column) ** $p < 0.01$ compared to the positive control group.

Also the serum level of IL-1β was decreased up to 48% in the group treated with *S. fruticosa* compared to the LPS-stimulated mice, reaching 5232 pg/ml, then falling to 2766 pg/ml ($p < 0.05$). There was no significant effect on the baseline level of IL-1β in the serum of the only extract-treated mice and the normal control group as shown in Fig.19

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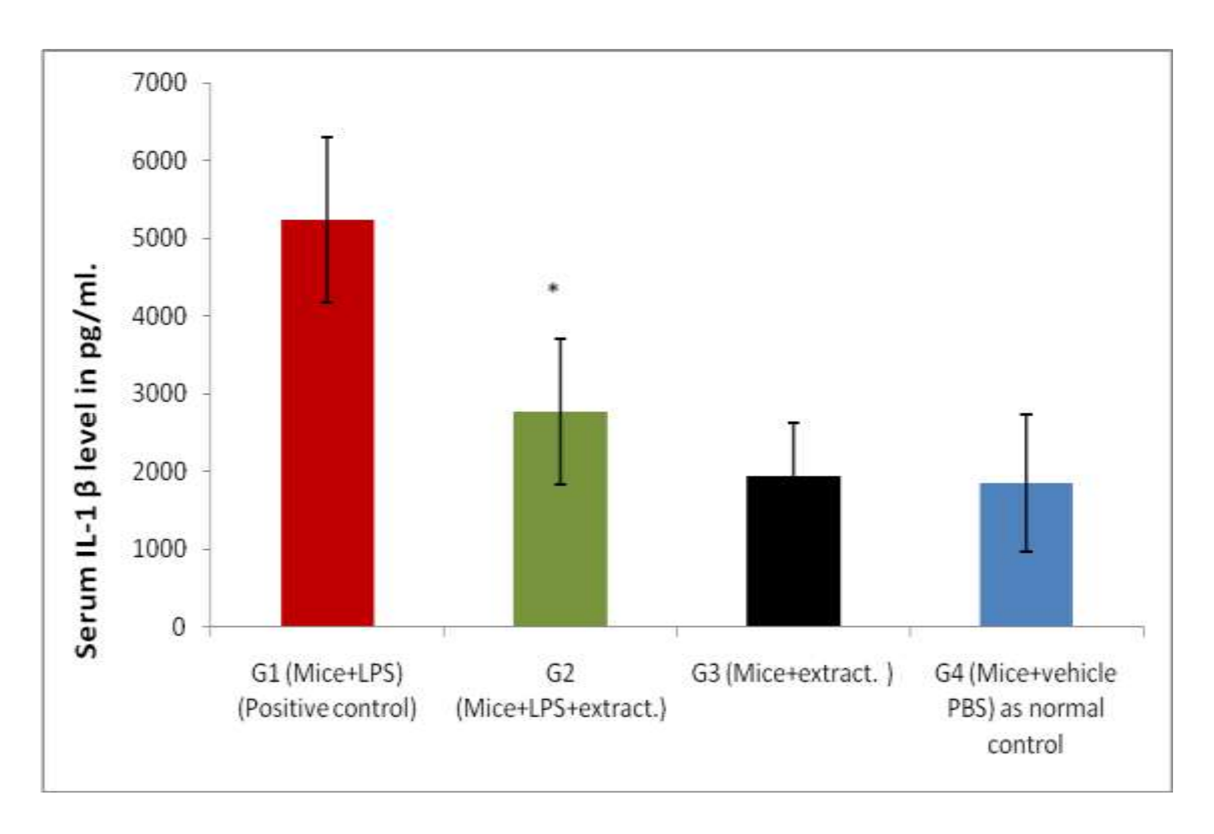


Figure 19. Effect of *S. fruticosa* on serum IL-1β of Balb/c mice. G1: mice only stimulated with LPS (red column); G2: mice treated with *S. fruticosa* extract and stimulated with LPS (green column); G3: mice only treated with *S. fruticosa* extract (black column); G4: mice only injected with the vehicle PBS (blue column) * $p < 0.05$ compared to the positive control group.

4.3. Effect of *S. fruticosa* on MLR *in vitro*

The *in vitro* MLR results as illustrated in Fig.20 showed an inhibitory effect of *S. fruticosa* on proliferation activity of splenocytes at the highest concentration of the extract used (250 µg/ml) and a p value < 0.01. Interestingly, this significant inhibitory effect was followed by a significant stimulation at extract concentration of 125 µg/ml compared to the R+S (positive control group) with a p value < 0.01. Then, returned to the level of the positive control group at the concentrations 62.5 and 31.25 µg/ml. As expected the stimulator (S) cells did not exhibit proliferation activity and the proliferation activity in the (R+S) group increased significantly compared to the normal group (R) with a p value < 0.01.

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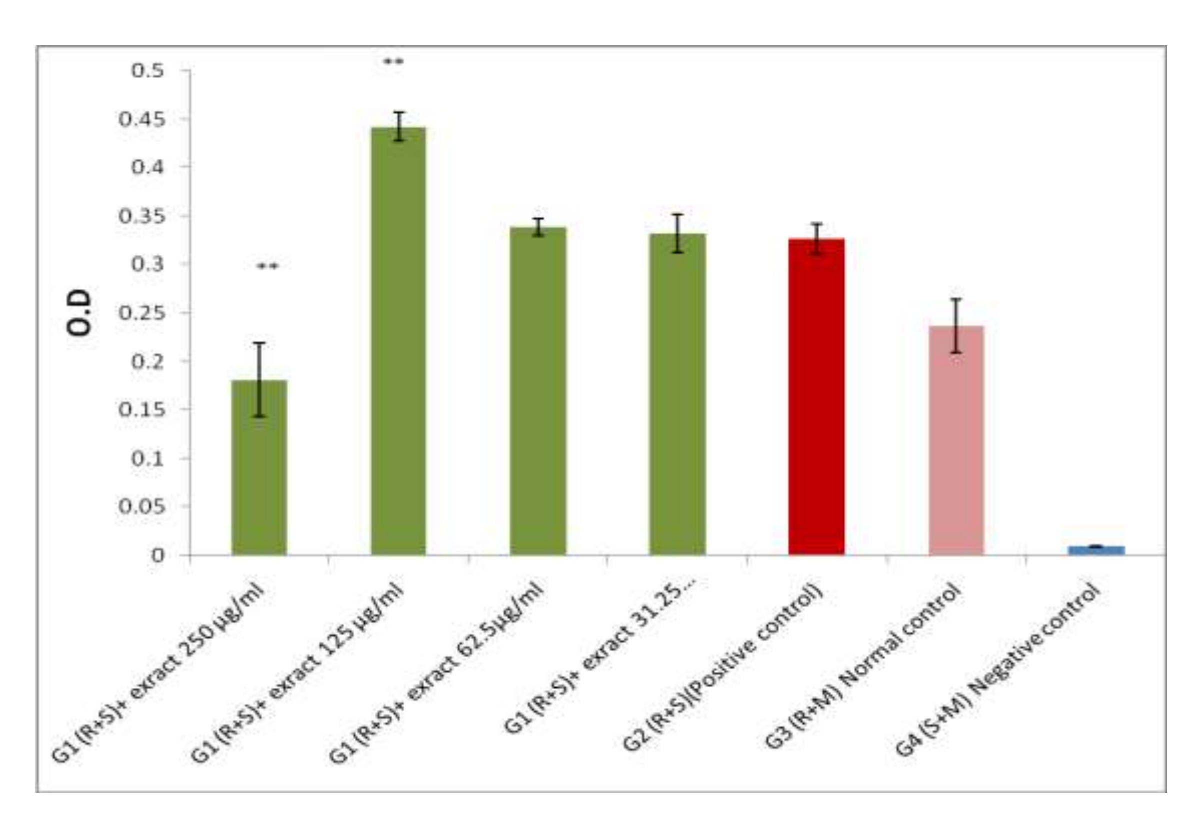


Figure 20. Effect of *S. fruticosa* on MLR *in vitro*. G1: mixed splenocytes (R+S) treated with different concentrations of the plant extract (green columns); G2: mixed splenocyes without *S. fruticosa* treatment (R+S) positive control group (red column); G3: mice splenocytes incubated wit medium only (R+M) normal control group (pink column); G4: rat splenocytes inactivated with mitomycin C and incubated with medium only (S+M) negative control group (blue column) ** $p < 0.01$ compared to the positive control group.

4.4. Effect of *S. fruticosa* on PIP3 production level in RAW 264.7 cells

This experiment was conducted to investigate the changes in PI3K activity in LPS-stimulated RAW 264.7 cells and the effect of the treatment of *S. fruticosa* extract on such changes by extraction of PIP3 from these cells and measuring its level by ELISA. The results demonstrated that *S. fruticosa* had strongly suppressed PIP3 production in cells treated with the plant extract before being stimulated with LPS at all extract concentrations used (31.25-250 µg/ml). The inhibition ratios reached between 83-85% regardless the concentration used, compared to the single-LPS stimulated cells (the positive control) reaching 602 pmol/ml and then dropped down to 84 pg/ml ($p < 0.05$).

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The positive control group increased PIP3 production by 10-folds compared to normal unstimulated cells. Moreover, there was no statistically significant difference in PIP3 level between the extract/LPS-stimulated group and the normal unstimulated cells as shown in Fig.21.

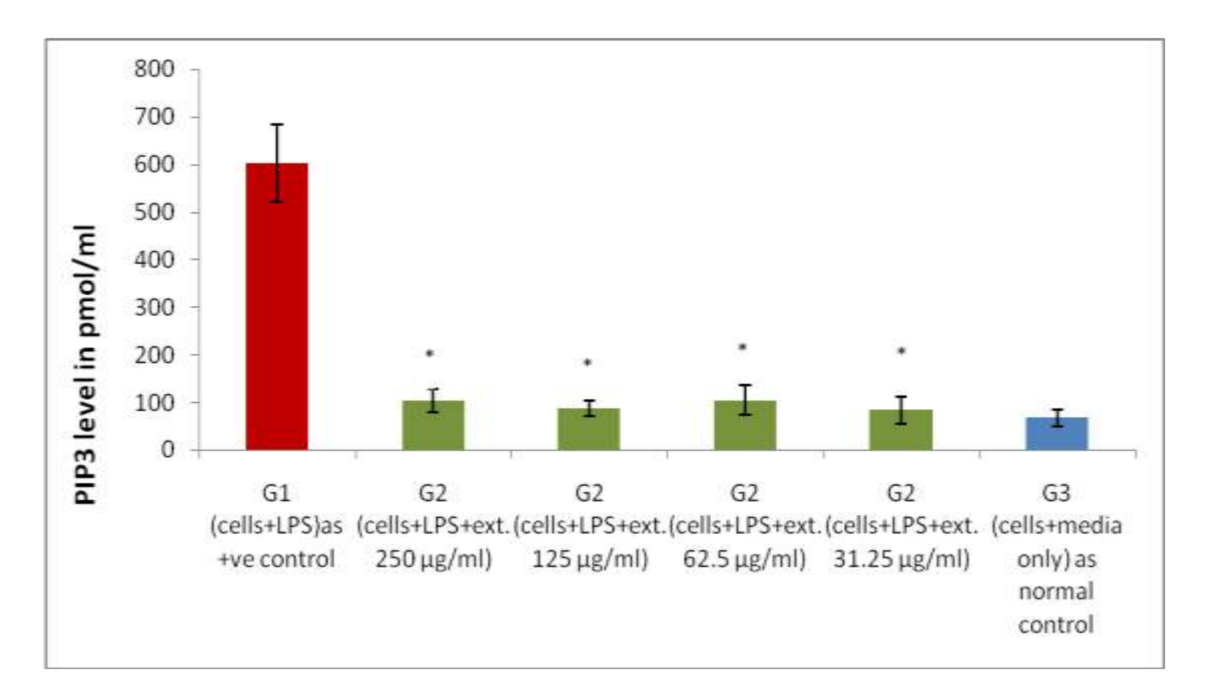


Figure 21. Effect of *S. fruticosa* on PIP3 production level in RAW 264.7 cells. G1: cells treated with *S. fruticosa* at different concentrations and stimulated with LPS (green columns); G2: LPS-stimulated cells, positive control group (red column); G3: cells incubated with medium only, normal control group (blue column) * $p < 0.05$ compared to the positive control group.

4.5. Effects of treatment with *S. fruticosa* on a disease model of adjuvant-induced arthritis in rats

4.5.1. Effects on clinical measures

The course of arthritis in animals injected with adjuvant was followed every other day by measuring the volume of both hind paws (milliliters, as an indicator of edema) using hydroplethysmometer. All measurements were obtained at the same time of the day. As illustrated in Fig.22A and table 1, on day zero (starting of induction and treatment), the

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right hind paw volume was almost the same in all groups. From day 2 onwards to day 14, there was a continuous increase of paw volume of the arthritic untreated group (disease group) and that the peak volume was on day 14, then it started decreasing until it returning approximately to the volume measured on the zero day. The *S. fruticosa* treated group and the normal group showed no significant changes (Fig.22B, Fig.22C and 22D). The difference was significant between the disease group and the other groups ($p < 0.01$). However, the swelling of the right paw started to appear on the second day after CFA injection, but the severity was critically observed on day 14 (Fig.22B).

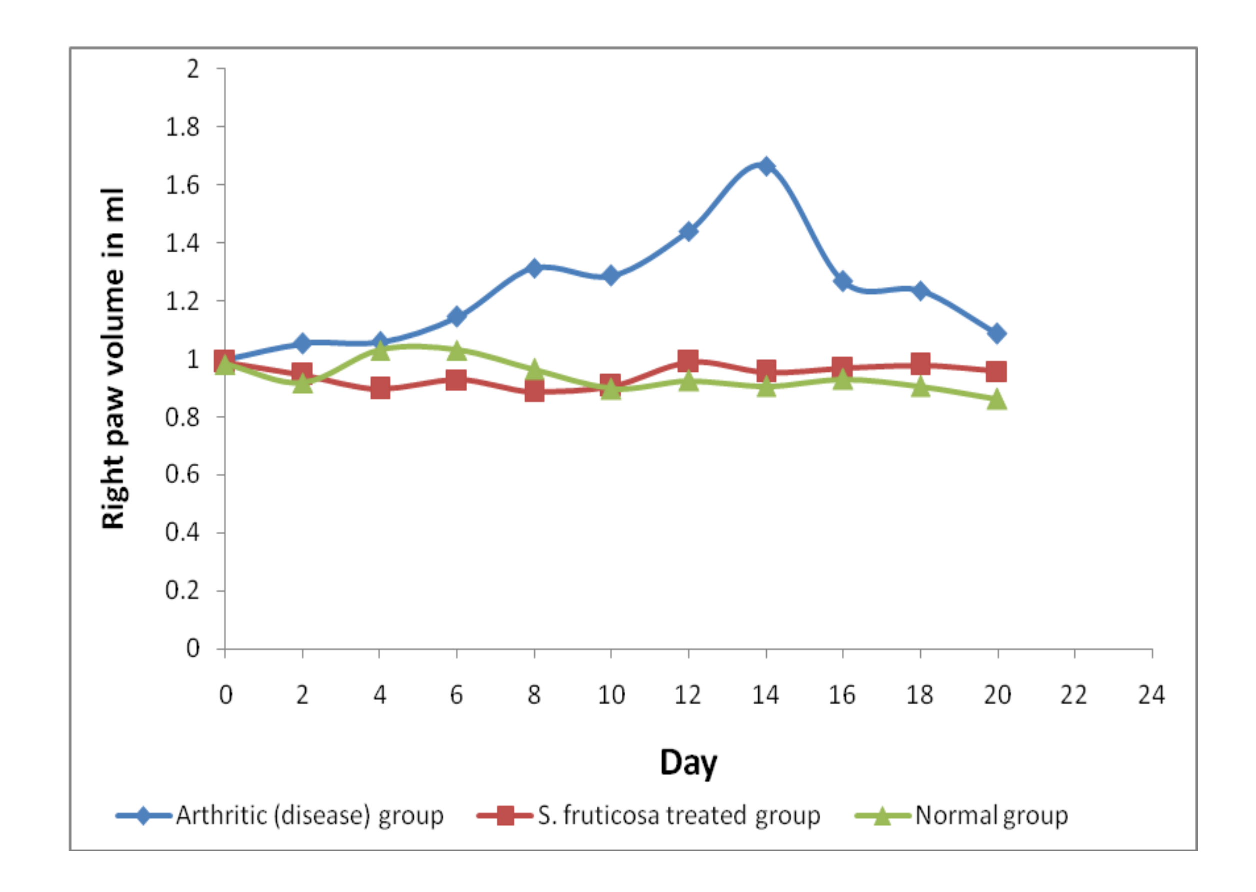


Figure 22A. Effects of the treatment with *S. fruticosa* on right hind paw volume in adjuvant-induced arthritis in rats.

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Table 1: Right hind paw volume in ml.

Right paw volume in ml			
Day	Arthritic (disease group)	*S. fruticosa* treated group	Normal group
0	0.99 ± 0.05	0.98 ± 0.06	0.98 ± 0.08
2	1.05 ± 0.08	0.95 ± 0.09	0.92 ± 0.07
4	1.05 ± 0.15	0.9 ± 0.16	1.03 ± 0.09
6	1.15 ± 0.18	0.93 ± 0.16	1.03 ± 0.09
8	1.31 ± 0.30	0.89 ± 0.09	0.96 ± 0.06
10	**1.3 ± 0.15**	0.9 ± 0.10	0.9 ± 0.07
12	**1.44 ± 0.07**	0.99 ± 0.12	0.93 ± 0.12
14	**1.67 ± 0.20**	0.95 ± 0.08	0.9 ± 0.12
16	1.26 ± 0.15	0.96 ± 0.14	0.93 ± 0.09
18	1.23 ± 0.16	0.97 ± 0.13	0.91 ± 0.08
20	1.08 ± 0.14	0.96 ± 0.16	0.86 ± 0.09

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Figure 22B. Right hind paw of arthritic (disease group) on day 14, swelling and redness of the paw and toes were clear.



Figure 22C. Right hind paw of *S. fruticosa* treated group on day 14, no swelling or redness of the paw and toes was observed.



Figure 22D. Right hind paw of normal group on day 14, no swelling or redness of the paw and toes was observed.

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The left paw volume start increasing from day 2 to 12 in the arthritic group reaching maximum in days 10 and 12, then decreased until approximately returned on day 20 to the volume measured on day zero. The *S. fruticosa* treated group and the normal group showed fluctuations in left paw volume measurements, but they were significantly less than the arthritic group ($p < 0.01$) and on the day 20 paw volume was less than the volume measured on the zero day of experiment (Fig. 23A, 23B, 23C and 23D). Also table 2 showed the left paw volume through 20 days of experiment.

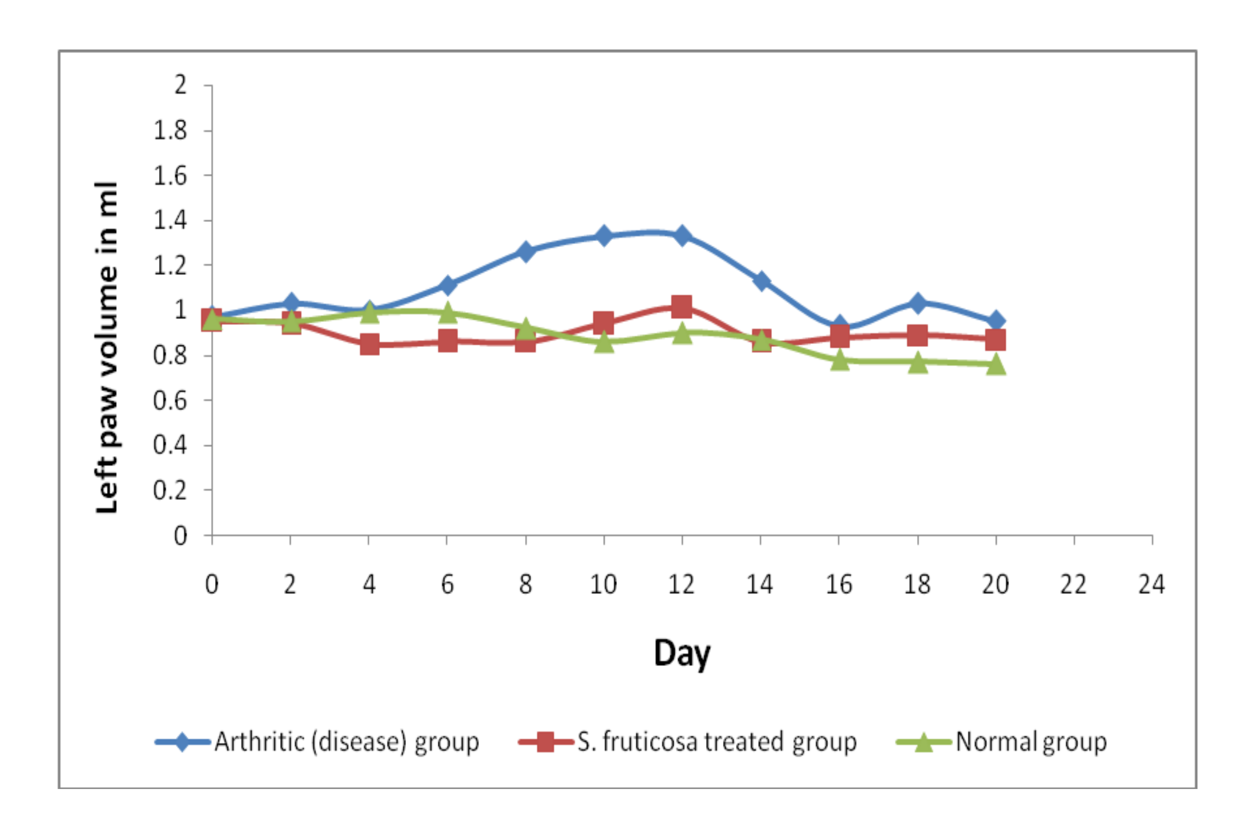


Figure 23A. Effects of the treatment with *S. fruticosa* on left hind paw volume in adjuvant-induced arthritis in rats.

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Table 2: Left hind paw volume in ml.

	Left paw volume in ml		
day	Arthritic (disease group)	*S. fruticosa* treated group	Normal group
0	0.97 ± 0.06	0.95 ± 0.09	0.96 ± 0.06
2	1.03 ± 0.08	0.94 ± 0.11	0.95 ± 0.08
4	1.00 ± 0.11	0.85 ± 0.18	0.99 ± 0.11
6	1.11 ± 0.20	0.86 ± 0.11	0.99 ± 0.11
8	1.26 ± 0.30	0.86 ± 0.08	0.92 ± 0.13
10	1.33 ± 0.23	0.94 ± 0.07	0.86 ± 0.08
12	1.33 ± 0.23	1.01 ± 0.09	0.9 ± 0.07
14	1.13 ± 0.07	0.86 ± 0.16	0.87 ± 0.08
16	0.93 ± 0.09	0.88 ± 0.15	0.78 ± 0.15
18	1.03 ± 0.22	0.89 ± 0.15	0.77 ± 0.09
20	0.95 ± 0.18	0.87 ± 0.14	0.76 ± 0.12

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Figure 23B. Left hind paw of arthritic (disease group) on day 14, swelling and redness of the paw and toes are less clear than in right paw and as compared to other groups.



Figure 23C. Left hind paw of *S. fruticosa* treated group on day 14, no swelling or redness of the paw and toes was observed.



Figure 23D. Left hind paw of normal group on day 14, no swelling or redness of the paw and toes was observed.

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In agreement with the inhibition of paw volume, treatment with *S. fruticosa* also inhibited clinical manifestations of arthritis in rats treated with *S. fruticosa* compared to the arthritic group ($p < 0.01$). Since arthritic scores in all rats of the normal group remained zero for the duration of the experiment, it was not shown in Fig. 24. Also the clinical manifestation of the treated group was close to the normal group. In contrast, it was observed that the incidence of the disease was 100% only in the arthritic group with totally sever manifestations observed from day 12 and it remained as such throughout the duration of the experiment except on day 24 severity was less compared to the peak period, but it still more obvious than at starting the experiment shown in Fig. 24 and table 3.

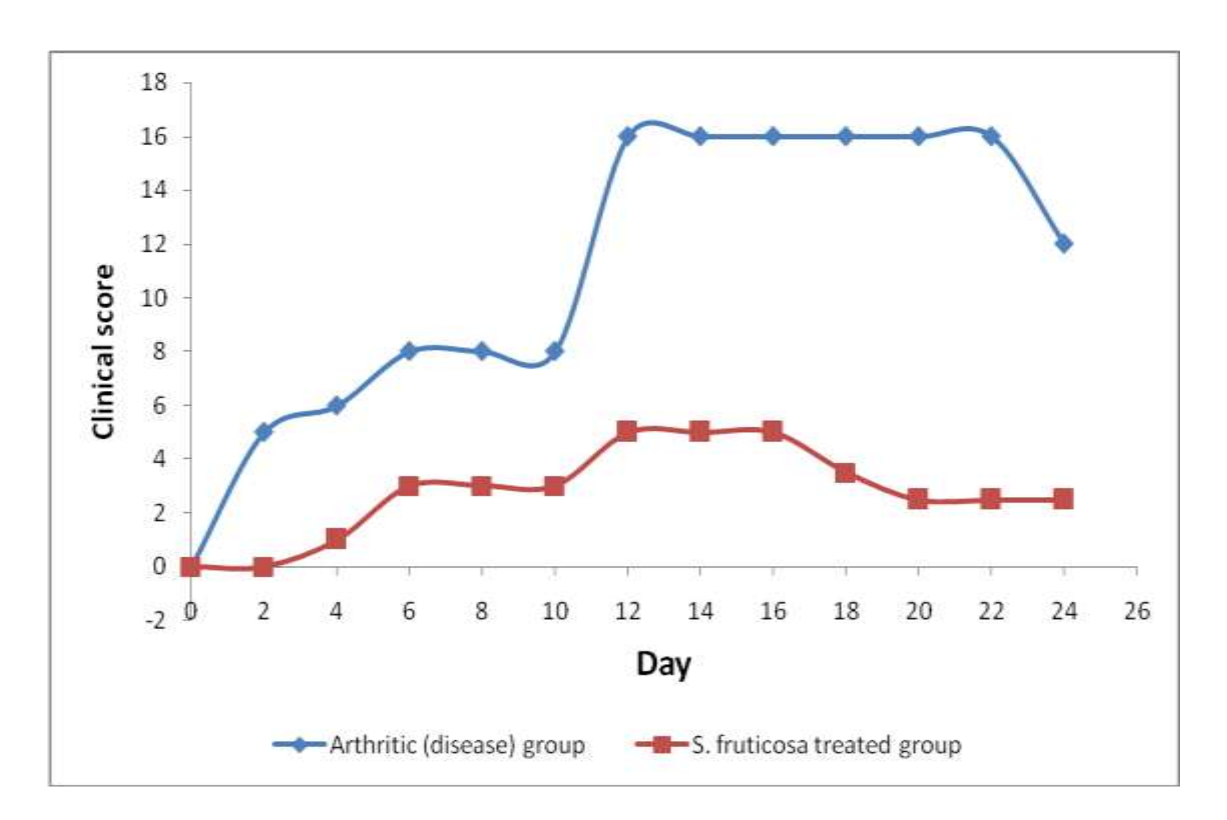


Figure 24. Effects of the treatment with *S. fruticosa* on clinical scoring in adjuvant-induced arthritis in rats.

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Table 3: Clinical score of AIA

Clinical scoring of AIA		
days	**Arthritic group (disease)**	***S. fruticosa* treated group**
0	0	0
2	5	0
4	6	1
6	8	3
8	8	3
10	8	3
12	16	5
14	16	5
16	16	5
18	16	3.5
20	16	2.5
22	16	2.5
24	12	2.5

Results of the radiological pictures of the joints of the arthritic group and treated group done on day 17 of the experiment showed striking differences as reported by an expert, board-certified radiologist. There was subchondral bone sclerosis, decreased joint space definition and soft tissue swelling in the arthritic group, which are less marked in the group that was treated with *S. fruticosa*. Also, X-ray was done for the normal group for comparison (Fig. 25A and 25B) for arthritic group, (Fig. 26A) and (Fig.26B) for *S. fruticosa* treated group. (Fig. 27A) and (Fig. 27B) for normal control group.

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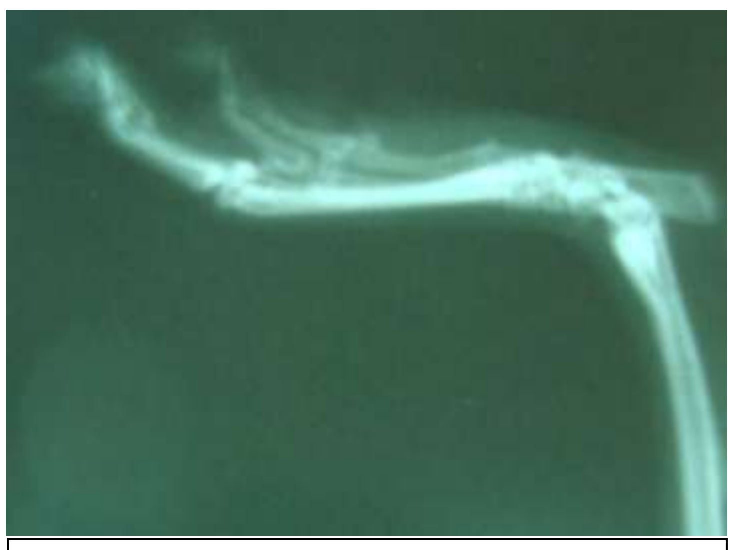


Figure 25A. Radiographic image of a paw from arthritic group.



Figure 25B. Radiographic image of a paw from arthritic group.



Figure 26A. Radiographic image of a paw from *S. fruticosa* treated group.

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Figure 26B. Radiographic image of a paw from *S. fruticosa* treated group.

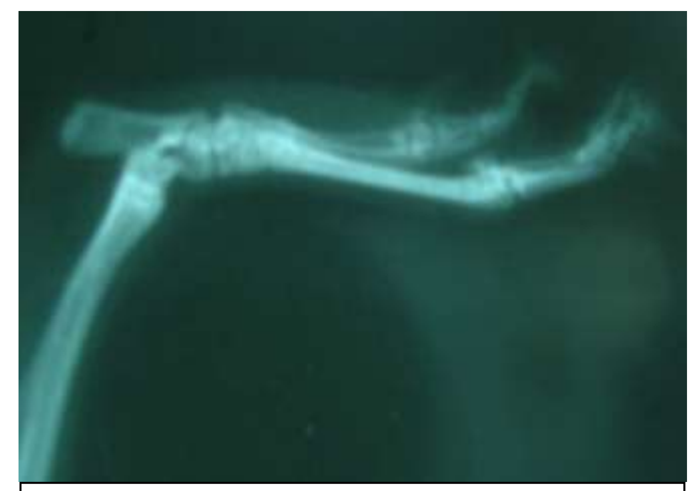


Figure 27A. Radiographic image of a paw from normal control group.



Figure 27B. Radiographic image of a paw from normal control group.

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4.5.2. Effects on behavior

Moreover, adjuvant-induced arthritic rats were not only observed clinically, but also behaviorally. Our concern in investigating the behavioral changes in this experiment was additional to confirm the successful establishing of the disease model by integrating many parameters related to RA.

It has been known that the inflamed tissues that so develop are hyperalgesic (Colpaert, 1987). Based on the literature and previous observations of normal rats, the following behavioral elements were used as parameters for the quantification of the behavior: rearing, sniffing, grooming, scratching and freezing (Costa *et al*, 1981).

Rearing means that the rat “looks for” something in the air. For this, it elevates its head and forepaws, almost standing up, and vibrates its snout whiskers, sometimes swinging its body.

Sniffing consists of nosing on the floor. The rat holds the head down and at the same time, walks or runs.

Grooming means licking parts of the body or sometimes rubbing the ears, the head or the snout with the forepaws.

Scratching implies different actions which occur as a coherent behavioral pattern. Usually the rat starts by licking some parts of the body but changes to biting the skin or sometimes the rat also scratches by rapidly vibrating the elevated hindpaws near or on its ears.

Freezing is also a complex behavior. It implies a state of staring, looking attentively at something somewhere. Freezing sometimes alternates with sniffing, rearing, eating, drinking, as a sudden stop in those activities. The rat adopts an immobile position, its snout whiskers become less mobile.

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The arthritic rats showed a clear increase of scratching, rearing and freezing compared to the *S. fruticosa* treated group and the normal control group. These behavioral elements were video recorded.

There are also other behavioral elements such as climbing, eating, drinking, resting and sleeping but they were not monitored in this study and require no description.

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5. Discussion

The host defense response to pathogens depends on the immune system. The immune system in mammals consists of innate and adaptive immunity. Adaptive immunity is a highly sophisticated system that is mediated by antigen-specific T and B cells, and is observed only in vertebrates. In contrast, innate immunity is conserved from invertebrates to vertebrates. Even invertebrates harbouring only innate immunity have an effective host defense system. The mechanism for recognition of non-self (antigen) by adaptive immunity has been investigated intensively and the major mechanisms have been clarified. However, the mechanism for innate immune recognition of non-self has long remained unclear ((Takeda and Akira, 2001). Studies of the host defense system in fruit flies provided the first clue as to the mechanism of innate immune recognition. In *Drosophila*, a family of Toll receptors plays an important role in combating the invasion of pathogens. Subsequently, homologues of *Drosophila* Toll were identified in mammals, and are now termed Toll-like receptors (TLRs) which compose a large family with at least 10 members. Evidence is accumulating that TLRs play an important role in the recognition of components of pathogens and subsequent activation of innate immunity, which then leads to development of adaptive immune responses (Medzhitov and Janeway, 2000). Although, innate immunity was thought for a long time to be non-specific and, consequently, research into innate mechanisms took second place to research into adaptive immunity. In recent decades, innate immunity is now at the forefront of current immunological research and that innate and adaptive immunity now have the same relevance (Coutinho and Poltorack, 2003).

The capability of the mammalian immune system to respond to a variety of threats is not just a simple alarm system for triggering antigen-presenting cells and initiating cellular immunity. Instead, the body is an integrated system in which nearly every cell

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type can relay the alarm through the production of chemokines, which recruit specific inflammatory cells to the target tissues. This integration of innate and adaptive immunity allows for both rapid responses and dynamic regulation of inflammation *in vivo* (Lo *et al*., 1999). So, in view of this interplay between several components of the immune system we examined the inhibitory effects of *S. fruticosa* on several potential targets that supposed to participate in a way or another in development and progression of rheumatoid arthritis by using different models including: *in vitro*, *in vivo* and finally the disease model in this research.

5.1.1. Inflammatory model establishment and procedure monitoring

The cytokines TNF-α, IL-1β and IL-6 are known to be pro-inflammatory cytokines that possess a multitude of biological activities linked to the immunopathology of acute or chronic inflammatory diseases such as autoimmune diseases, of which, RA, is one of the most common. There are a lot of *in vitro* and *in vivo* studies demonstrated that medicinal plants modulate the secretion of multiple cytokines.

Phytotherapy offers a potential therapeutic pattern for the treatment of many differing conditions involving cytokines that play major roles and have broad-spectrum effects on progression of autoimmune conditions and chronic degenerative processes, and so, modulation of cytokine secretion may offer novel approaches in the treatment of a variety of these diseases (Spelman *et al*., 2006).

It was demonstrated that phenolic contents present in higher amounts in the methanol extract than the aqueous extracts and exhibit a stronger antioxidant activity due the fact that methanol is less polar solvent than water which implied that methanol is more efficient solvent for cell wall leading to the release of polyphenols from cells. Moreover, it is believed that the activity of polyphenols oxidase which degrades

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polyphenols in water extracts is neutralized in methanol medium (Al-Mustafa and Al-Thunibat, 2008).

Hence, we examined the effect methanolic crude extract of *S. fruticosa* on these pro-inflammatory cytokines production by RAW 264.7 cells, peritoneal macrophages, BMDM and splenocytes using ELISA method.

As shown from figures on pro-inflammatory cytokines in the results section of this thesis, the levels from cells stimulated with LPS in the case of RAW 264.7 cells, BMDM, peritoneal macrophages and from the serum of Balb/c mice and ConA in the case of splenocytes were significantly higher than those of normal cells, which implied the successful establishment on model of inflammation. Meanwhile, the levels of those cytokines from cells by *S. fruticosa* intervention was significantly lower than those from single LPS or ConA stimulation. The effects of *S. fruticosa* intervention demonstrated experimental procedure was proper as well as with the PIP3 production level in RAW 264.7 cells.

Moreover, the reduced levels of those cytokines were due to the *S. fruticosa* intervention rather than cytotoxic effect of the plant that might be proposed, because viabilty of all cell types used in this research was not affected by treatment of these cells with the plant extract at various concentrations as examined by MTT assay and under the same conditoions of cytokines production experiments.

5.1.2. Inhibitory effects of *S. fruticosa* on pro-inflammatory cytokines production by LPS-stimulated RAW 264.7 cells

Overproduction of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 play an important role in the inflammatory process and they are defined as “ alarm cytokines” that are secreted by macrophages and initiate inflammation (Krishnaveni and

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Jayachandran, 2009). In experimental practice, RAW 264.7 mouse macrophage cell line stimulated by LPS is widely used as the inflammatory cellular model to study the effect of anti-inflammatory drugs and herbs (Zhao *et al*., 2007).

The results obtained from this experiment demonstrated strong inhibitory effect of *S. fruticosa* on pro-inflammatory cytokines production by LPS-stimulated RAW 264.7 cells, especially TNF-α and IL-6. The differential effects of *S. fruticosa* extract on TNF-α and IL-6 versus IL-1β might be due to differential effect of the extract on those pro-inflammatory cytokines as claimed by Singh *et al*., (2006). Also, this differential effect might be due to other factors that might correlate with the secretion mechanism IL-1β production in response to LPS.

5.1.3. Inhibitory effects of *S. fruticosa* on pro-inflammatory cytokines production by LPS-stimulated peritoneal macrophages

Macrophages isolated from murine peritoneal cavity, bone marrow and spleen are suitable samples for studying the activation properties of this immunologically important cell type. The peritoneal cavity provides an accessible site for harvest of fair numbers of resident macrophages. To increase macrophage yield, sterile inflammatory agents, such as proteose peptone or thioglycollate, can be injected into the peritoneal cavity prior cell harvest. Although these inflammatory agents increase the number of peritoneal macrophages present, concerns have been raised as to how inflammatory agents affect the activation state and contributte to heterogeneity of macrophages. However, such inflammatory induction will alter the physiologic characteristics of the cells collected. Generally, the normal mouse peritoneal cavity will yield mature, resident macrophages and the inflamed peritoneal cavity will yield immature,

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inflammatory macrophages recruited from the circulating and marginal pool (Donovan and Brown, 1995).

However, in this study we prefered. to isolate macrophages from noninflamed peritoneum.

The results of this experiment as illustrated previously, clearly showed an extremely tremendous levels of TNF-α and IL-6 in single LPS-stimulated cells with an extremely potent inhibition in *S. fruticosa* treated cells reaching up to 96-99.5%.

On the contrary, IL-1β level was minimal upon LPS stimulation, but in line with the results of RAW 264.7 cells IL-1β production experiment which deserve more investigations.

5.1.4. Inhibitory effects of *S. fruticosa* on pro-inflammatory cytokines production by LPS-stimulated BMDM

Also, continuing examination of the inhibitory effects of *S. fruticosa* on another macrophage cell type, the primary BMDM as another cellular model, since they represent a homogenous, non-transformed population of macrophages that can be stimulated *in vitro* to proliferate by macrophage colony stimulating factor (M-CSF) or activated by LPS.

The results obtained by using this type of cells was not incompitable with the results from the previous experiments on RAW 264.7 cells and peritoneal macrophages in which there was high levels of TNF-α and IL-6 in LPS-stimulated cells accompanying significant inhibition in *S. fruticosa* treated cells. On the other side, IL-1β level was slightly increased in this type of cells as well as the other cell types.

However, mechanisms of IL-1β secretion remain controversial, with several theories being proposed. It remains unclear whether differences in the functional state of the

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cells or in the cell culture conditions account for different secretory pathways chosen by these cells. A better understanding of the mechanisms involved in IL-1β processing and release is of great therapeutic interest and may help to develop strategies aimed at reducing the severity of inflammatory and autoimumune diseases (Eder, 2009).

5.1.5. Inhibitory effects of *S. fruticosa* on pro-inflammatory cytokines production by ConA-stimulated splenocytes

The spleen has two main functions acting as part of the immune system and as a filter . There are two distinct components of the spleen, the red pulp and the white pulp. The red pulp consists of large number of sinuses and sinusoids filled with blood and is responisible for the filtration function of the spleen. The white pulp consists of aggregates lymphoid tissue and is responsible for the immunological function of the spleen. The white pulp contains T cells, B cells and accessory cells.

In the present study, we have determined the inhibitory effects of *S. fruticosa* on pro-inflammatory cytokines production by splenocytes as a lymphocyte cellular model, maily, T cells. Because in many experiments (data not shown) we tried LPS in addition to ConA for evaluating splenocyte stimulation and proliferation. Splenocytes did not respond to LPS stimulation and only respond to ConA and these observations were identical to results obtained in a study conducted by Devi and coworkers in 2003. ConA binds to to the T-cell receptor/CD3 complex and activates lymphocytes largely via phospholipase-C-mediated events. LPS binds to the surface receptor protein CD14, which is not found on T cells but is found on B cells. Thus, ConA stimulates T-lymphocytes, whereas LPS stimulates B lymphocytes (Devi *et al.*, 2003).

Although there was inhibitory effect of *S. fruticosa* on its production, its level in ConA-stimulated splenocytes was less than two fold increase, which may be explained as

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lymphocytes are not major producer of this inflammatory cytokine and this observation is in line with literature that macrophages are the main producer of this key cytokine.

The production level of IL-6 was significantly increased in ConA-stimulated splenocytes compared to unstimulated cells and markedly inhibited at all extract concentrations used, except the lowest one (31.25 µg/ml).

As in the previous experiments on macrophages, IL-1β production did not reach high levels in ConA-stimulated splenocytes, its increase was less than 2 fold compared to the normal cells. Eventhough, this increase was statistically significant and that the ability of *S. fruticosa* treated splenocytes was significantly diminished to produce IL-1β at all concentrations of the extract used.

5.2. Effect of *S. fruticosa* on splenocytes MLR

The mixed lymphocyte reaction (MLR) was performed to determine the ability of mice splenocytes (responder cells) to recognize allogeneic rat splenocytes (stimulator cells) that were inactivated by mitomycin C and proliferate as a consequence of this recognition as described by Thorpe and Knight (1974). The activating stimuli is the foreign histocompatibility antigen, usually, the major histocombatibilty complex(MHC) class I or class II molecules expressed on the stimulator cells. The MLR was conducted as an indicator of cell-mediated immunity and studying the effect of *S. fruticosa* on such type of immunity.

The results of this experiment showed different phases of proliferation activity of splenocytes. The highest concentration of the plant extract used (250 µg/ml) significantly inhibited splenocytes proliferation, and then, followed by an activation at concentration 125 µg/ml. Then, returned to the level of the positive control group (R+S)

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at concentrations 62.5 and 31.25 µg/ml which means no effect of *S. fruticosa* at these two concentrations.

5.3. Effects of *S. fruticosa* treatment on pro-inflammatory cytokines production *in vivo*

Results of *in vitro* assays provide important and useful information, but do not necessarily reflect *in vivo* cytokine secretion. In addtion, all of these assays examine cytokine production by a single organ rather than the whole animal (Finkelman *et al*., 2003).

The experiments codnducted on cellular models in this reasarch to evaluate the inhibitory effect of *S. futicosa* on the production of pro-inflammatory cytokines *in vitro* were promising, but need to be sudied *in vivo.*

Also, it is important to note that most of the research done on *Salvia* employs *in vitro*-based studies and *in vivo* tests should be encouraged (Kamatou *et al*., 2008).

Therefore we aimed in this study to evaluate the effects of treatment with *S. fruticosa* on production in LPS-stimulated mice.

Inhibitiory effects of *S. fruticosa* were clearly significant in suppressing the release of all cytokines examined and in contrast to *in vitro* assays results, IL-1β serum level was significantly increased in LPS-stimulated mice compared to the normal group.

This may be an example of difference between *in vitro* and *in vivo* conditions or could be explained by that the blood was not collected at the peak time of the IL-1β secretion *in vivo* .

There is a lot of studies investigating the time of peak levels of TNF-α, IL-6 and IL-1β secretion in Balb/c mice and other species. Serum TNF-α peaked at 1 or 1.5 hours after LPS injection and IL-6 showed higher peak levels 2-3 hours. Serum IL-1β peak leveles

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showed a wider range 2-6 hours after LPS injection (Bitzer-Quintero *et al*., 2005; Fraser *et al*., 2009; Hong *et al*., 2009). Therefore, overall, the time point of 2 hours after LPS injection was chosen for this experiment.

5.4. Effects of *S. fruticosa* on PI3K activity in LPS-stimulated RAW 264.7 cells

The production of PIP3 from PIP2 by type-1 PI3K is important in multiple cell signaling pathways. Typically, experiments to measur PI3K activity have involved phosphorylation of phosphoinostide substrate using ^{32}P, then extraction of radioactive products, and separation using thin-layer chromatography. The assay plate method developed by Echelon Biosciences, Inc. allows the user to determine PI3K activity by measuring the amount of PIP3 extracted from cells by means of standard ELISA format, eliminating the need for radioactivity, and thin layer chromatography (Gray *et al*., 2003).

The class I PI3Ks are the only enzymes that can use PIP2 as a substrate to synthesize PIP3 (Rameh and Cantley, 1999). The synthesis of PIP3 is rapid and transient, whereas PIP2 accumulates more slowly during stimulation and in most cases, the levels of PIP2 are rather stable even in response to agonist stimulation (Payrastre *et al*., 2001).

A role for PI3K in inflammatory responses and innate immunity has begun to emerge from studies of signaling by receptors of the IL-1R and TNFR superfamilies. The inhibitors of PI3K have been reported to block NF-κB activation triggered by IL-1, TNF-α and anti-CD40 (a TNF receptor family member). A variety of cell lines have been used to investigate the mechanism of PI3K activation and its role in NF-κB regulation downstream of the IL-1R, TNFR and related receptors (Fruman and Cantley,

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2002). Reddy *et al* (1997), have demonstrated that a p85 subunit interacts with the IL-1 receptor in response to IL-1, leading to NF-κB activation.

The transcription factor NF-κB controls the biosynthesis of many mediators involved inflammation such as IL-1, IL-6 and TNF-α. The expression of NF-κB is increased at sites of inflammation such as the rheumatoid synovium (Morel and Berenbaum, 2004).

This experiment was conducted to evaluate and emphasize the important role of *S. fruticosa* intervention in disrupting an imporatant signaling transduction pathway involved in the development and progression of RA.

It was clear that *S. fruticosa* inhibited the production of PIP3 in LPS-stimulated RAW 264.7 cells approximately to the level in unstimulated cells as shown in the results. Also, our findings indicated that PI3K did exhibit activation in response to LPS stimulation for several minutes in RAW 264.7 cells and these results were consistent with several other sudies that used RAW 264.7 cells and other cell types and with LPS stimulation or other stimulation techniques which confirmed that the PIP3 produced resulted from PI3K activation, since that this activation was sensitive to both standard PI3K inhibitors, wortmannin and LY294002 (Chen *et al*., 2002; Kang *et al*., 2003; Niswender *et al*., 2003; Salh *et al*., 1998).

5.5 Effects of treatment with *S. fruticosa* on adjuvant-induced arthritis in rats

Adjuvant-induced arthritis is a widely used arthritic model for testing and developing anti-arthritic and anti-inflammatory agents and due to the strong correlation between the efficiency of therapeutic agents in this model and in RA in humans. However, its extensive use is sometimes restricted due to the variations in the incidence and severity of the disease, and the narrow number of susceptible rat strains available. *Sparague*

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Dawley (SD) rats are considered to be moderate responder strain to AIA (Jawed *et al*., 2010). In this present study we used SD rats, and demonstrated, that following subcutaneous inoculation (at the base of the tail) with CFA suspension, the SD rats developed severe arthritis at 100% incidence. There is much interest in the discovery of drugs that modify the progression of tissue destruction in RA. Here, we showed that treatment with *S. fruticosa* was efficient in preventing fundemental pathological processes in the joints of treated rats compared to untreated arthritic group.

The radiological results were of particular interest in this study. Narrowing of the joint space secondary to articular cartilage is diffuse within joints. The X-ray appearance, commonly referred to as diminshed or decreased joint space, is the hallmark of arthritis. The diminshed joint space represents a loss of articular cartilage, which may be brought about by a variety of pathological mechanisms (Ramprasath *et al*., 2006). Our radiological results showed an apparent decreased joint space in the arthritic group but not with the treated group.

Flavonoids have been shown to be responsible for preventing oseoporosis where they increase the bone mineral density (Nijveldt, *et al*., 2001). Flavonoids are widely utilized in medicine as antiosteoporotic agents. Experimental studies showed that flavonoids act by inhibiting osteoclastic bone resorption both *in vitro* and *in vivo* (Yamazaki *et al*., 1986). Flavonoids have been reported to bring benefits in lowering inflammation and oxidative stress and exert positive effects in cancer, cardiovascular and chronic inflammatory diseases (Aggarwal and Shishodia, 2006). It is well known that *Salvia* plants rich in flavonoids and other secondary metablites. From the results of the present study, it can be concluded that the strong anti-inflammatory and anti-arthritic activity exerted by *S. fruticosa* might be due to different components of its crude extract acting

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synergestically on more than one target in disease process such as pro-inflammatory cytokines, signal transduction pathways and bone metabolism.

Moreover, besides its widely use as a model for studying inflammation. It is reasonable to accept that AIA is painful, and therefore one might investigate whether this experimental pathological condition can be used as a model of somatic (as opposed to neurogenic) chronic pain (Costa *et al*., 1981).

In the medical literature, several reports are found on substances which have already been tested in the induced arthritis in rats, either for its ant-inflammatory or for its analgesic effects (Andersen *et al*., 2004). Taken together, results of this study strongly suggest that *S. fruticosa* is a highly potential candidate for well-covering all those purposes.

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6. Conclusions and recommendations

6.1. Summary and conclusions

The present work aimed to study the potential anti-rheumatic activity of *S. fruticosa* by evaluating the effects of its methanolic crude extract on different cell types, molecules and animal models. The results of this study clearly show that:

1. *S. fruticosa* caused significant decrease of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in all cell types used as an *in vitro* cellular models and in Balb/c mice as an *in vivo* animal model.

2. *S. fruticosa* potently interfers with a possible signal transduction pathway via inhibiting the activity of PI3K enzyme activity that involved in development and progression of RA.

3. More significantly, and as a proof of concept, *S. fruticosa* prevented the disease in the rats induced for adjuvant arthritis but treated with *S. fruticosa* as evaluated by clinical manifestations and paw volume measurements and finally proved by radiological study which demonstrated an exteremly significant differnces between the treated group and the arthritic untreated group.

4. In light of the adverse events experienced with cytokine-targeted therapy, it could prove useful to consider the use of phytotherapy in the modulation of cytokine expression. This study provides a scientific approach regarding the effects of *S. fruticosa* on many targets proposed to play vital roles in RA. In terms of drug efficacy though, as the history of crime fighters attests, good clinical benefits may require all " parteners in crime" to be neutralized.

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6.2. Recommendations

1. Because of the curative potential of this plant, efforts should be oriented towards its inclusion in the establishment of a Jordanian Pharmacopoeia where research related to the pharmacological and toxicological effects of this plant could be done.

2. More data are needed regarding selective inhibition of PI3K isoforms by *S. fruticosa*. Despite the tremendous effort already expended by the pharmaceutical industry, there are no reports of PI3Kγ-specific compounds progressing to clinic.

3. Further research activities following this line of evidence presented in this study will share in an extraordinary therapeutical, social, and economical impact.

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تقييم الفاعلية المضادة للروماتيزم لنبات الميرمية (العائلة الشفوية)، بإجراء دراسات على المستوى الأنزيمي و الخلوي و في الأجسام الحية

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ملخص

إن مرض التهاب المفاصل و الروماتيزم من الأمراض المزمنة و المستعصية و التي يعاني منها الملايين من الناس في مختلف أنحاء العالم، و من الممكن أن يؤدي هذا المرض إلى كثير من المضاعفات السلبية على صحة المرضى وقدرتهم على الحركة الطبيعية. لا يوجد سبب واضح وحيد ومتفق عليه لنشوء هذا المرض ولكن هنالك عدة فرضيات مقترحه لتفسير أسباب حدوث المرض وتطوره لكنها أيضا مثار جدل ونقاش في المجتمع العلمي والطبي. منها ما يتعلق بخلل في جهاز المناعة الذاتي أو ما يتعلق باستجابة جهاز المناعة لعوامل خارجية مثل الالتهابات الجرثومية أو أي عوامل أخرى منفردة أو مجتمعة. ما تم إثباته حتى الآن بالدراسات والأبحاث السابقة وطرق العلاج المتبعة حتى الآن أن هنالك مواد بروتينية تفرز بواسطة خلايا جهاز المناعة أو الخلايا الجسمية الأخرى معروفة بوسائط التهابية وأن هنالك آليات لنقل الإشارات الالتهابية داخل الخلايا ومفاقمة الوضع المرضي سوءا. لذلك أصبحت هذه العوامل الالتهابية أو الآليات التي تؤدي إلى تطور المرض محل بحث واسع ومكثف كأهداف محتملة للعلاج. إن الأدوية المستخدمة حاليا في معالجة أمراض التهابات المفاصل والروماتيزم باهظة الثمن وكذلك تحمل قدرا مهما من الآثار السلبية وحيث أن هؤلاء المرضى يستخدمون هذه الأدوية مدى الحياة وأن هذه الأدوية لم تعالج المرض نفسة بل المضاعفات الناتجة عن تطوره. لذلك فإن البحث عن أدوية مبتكرة ضد الروماتيزم يستمر وخاصة من المصادر الطبيعية كالنباتات وفي هذا البحث قمنا بدراسة

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للمستخلص الخام من الميرمية اليونانية أو ميرمية البحر الأبيض المتوسط والتي من المعروف أنها تستعمل في الطب الشعبي لمعالجة الكثير من الأعراض المرضية المختلفة منذ زمن طويل على أنماط خلوية متعددة من فئران التجارب شملت أنماط خلوية خبيثة وأنماط خلوية طبيعية. وكذلك على فئران حية لدراسة الأثر المثبط للميرمية على إنتاج وإفراز الوسائط الالتهابية في هذه النماذج المختلفة. كذلك وسعنا مجال الدراسة لتشمل أحد الأنزيمات المهمة في آلية نشوء المرض وتطوره لدراسة الأثر المثبط للميرمية على نشاط الأنزيم. وأخيرا تم إحداث التهاب روماتيزمي لدى الجرذان باستخدام (Complete Freund's adjuvant) كنموذج معترف به في مجال البحث العلمي والطبي أنه مشابه لمرض الروماتيزم عند الإنسان ويستخدم هذا النموذج لدراسة المرض نفسه أو اكتشاف وتقييم فاعلية الأدوية المضادة للالتهابات والروماتيزم. تم تجهيز مستخلص الميثانول من الأوراق المجففة للميرمية وتم فحصها على خلايا طبيعية من الفئران هي: خلايا التجويف البطني وخلايا الطحال وكذلك خلايا تم تكثيرها وتخصصها من الخلايا الجذعية للنخاع العظمي. أما النمط الخلوي الخبيث الذي تم استخدامه هو (RAW 264.7 cells) النتائج أظهرت بشكل مهم ومدهش الأثر التثبيطي الكبير للميرمية على إفراز العوامل الالتهابية سواء في النماذج الخلوية المختلفة أو في الفئران الحية حتى وصلت نسبة التثبيط في بعض هذه العوامل إلى ٩٩%. نتيجة أخرى عالية الأهمية وهي أن نشاط الأنزيم قد انخفض بشكل كبير حيث أن نسبة المادة الناتجة عن تفاعل هذا الأنزيم قد انخفضت بنسبة ٨٥% عند معالجة الخلايا بالميرمية. أخيرا في النموذج المرضي لدى الجرذان أظهرت الميرمية الفاعلية الكبيرة في عدم حدوث المرض عند الجرذان التى تم معالجتها بالميرمية بل لم تكن هنالك فروقات مهمه لدى مقارنتها بجرذان طبيعية غير مريضة استخدمت كمجموعة مراقبة ومقارنة للتجربة. في حين أن الجرذان التي لم تعالج أظهرت الأعراض السريرية الواضحة للمرض وعدم القدرة على الحركة الطبيعية وكذلك زيادة واضحة في قياس حجم القدم نتيجة للتورم. نتائج الصور الشعاعية للجرذان أظهرت بشكل واضح وجود تصلب في نسيج تحت الغضروف العظمي وتورم الأنسجة الرخوة وكذلك نقصان في المساحات بين المفاصل لدى الجرذان غير المعالجة وهذه هي العلامات البارزة لحدوث مرض الروماتيزم والتي لم تلاحظ عند الجرذان المعالجة بالميرمية. من نتائج هذه الدراسة نستنتج أن نبتة الميرمية تفيد في علاج الروماتيزم بتأثيرها على عدة عوامل في هذا المرض ولكن المزيد من الأبحاث المخبرية أو السريرية مطلوبة لتحديد القيمة العملية الفعالة والآمنة للتطبيق العلاجي على الإنسان.

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